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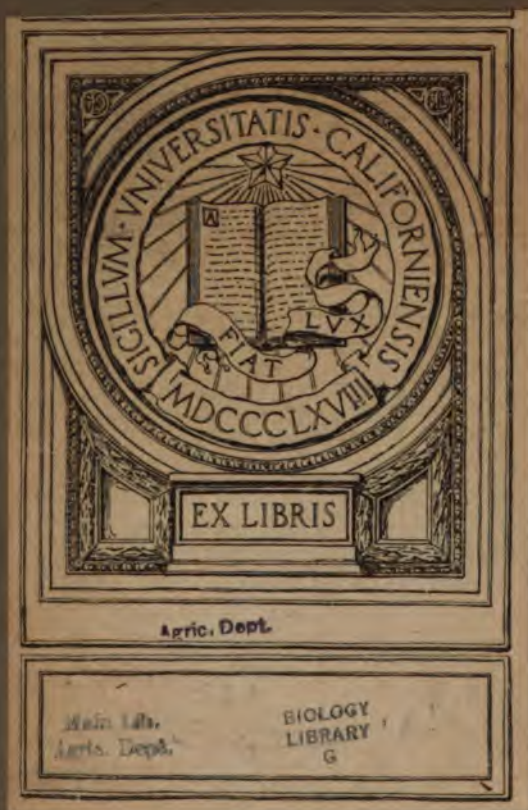


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IN BACTERIOLOGY

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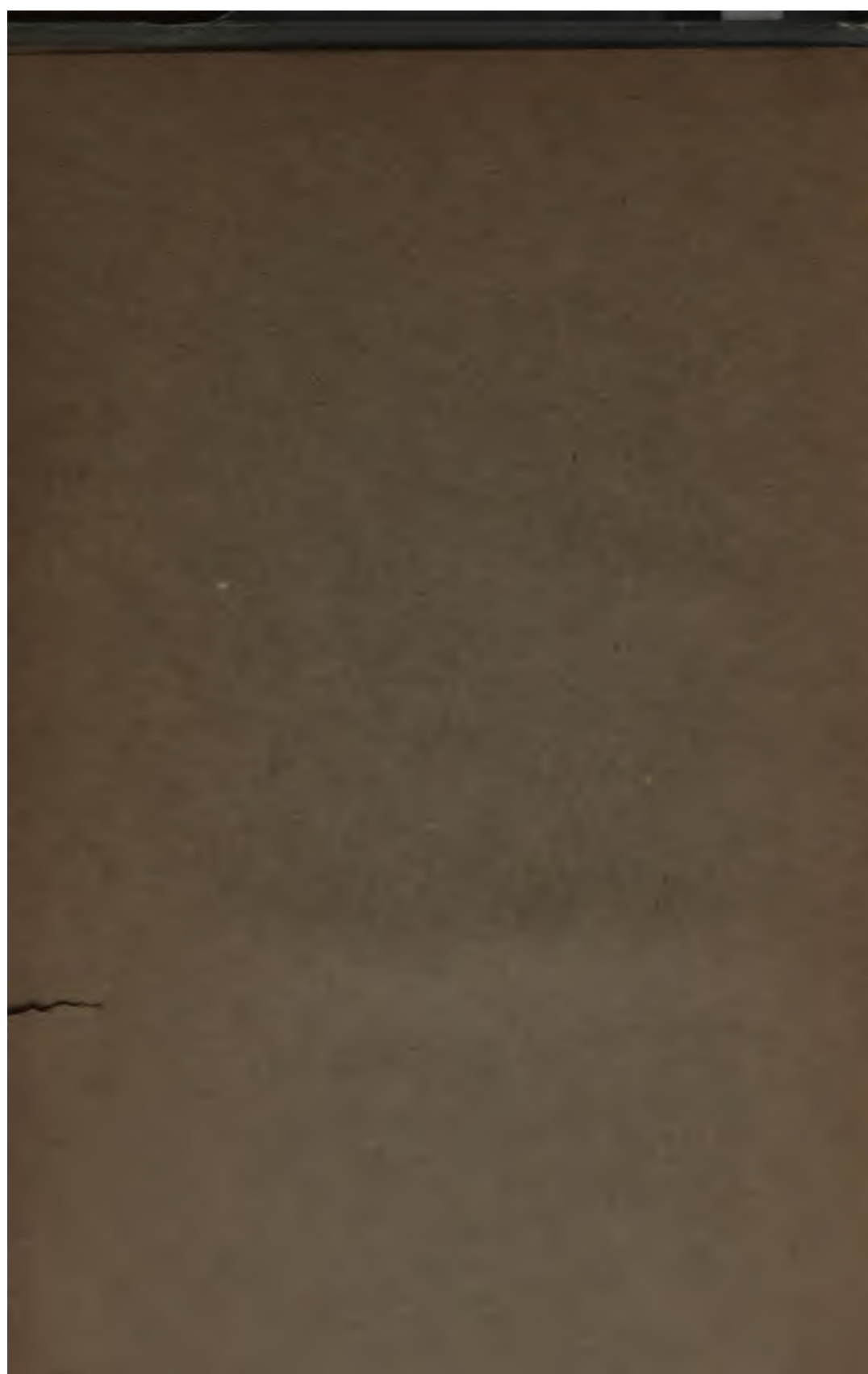


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LESSONS AND LABORATORY EXERCISES
IN BACTERIOLOGY.

SMITH.

LESSONS AND LABORATORY
EXERCISES

IN

BACTERIOLOGY

AN OUTLINE OF TECHNICAL METHODS INTRODUCTORY TO THE SYSTEMATIC STUDY AND IDENTIFICATION OF BACTERIA,

ARRANGED FOR THE USE OF STUDENTS

BY

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PREFACE.

The following pages were originally arranged as a series of exercises to be carried out by the class in the University of Texas under the guidance of an assistant, the hours of work of the several classes so overlapping that it was impossible for the writer to give his personal attention to the class in this laboratory, for the work in which he was, however, responsible. The exercises were outlined daily upon the blackboard, and verbal instructions as to their purpose and the manner of procedure given, as required, to the class. The arrangement being found well adapted to systematic work, it has been continued, with changes from and some additions to the original scheme, for several years; and recently, at the request of some of his students and with the thought that perhaps others might find some such definite arrangement of work of use in teaching, the writer has written out these exercises for publication. It has seemed advantageous that in their published form there should accompany the practical exercises such explanatory matter as would adapt the book as a student's laboratory outline guide. It would be beyond the writer's intention to make the work a compendium of methods, and the instructions, although forming a considerable bulk of the volume, include only such as from his experience he has adopted as best suited in class work.

There are arranged blank pages upon which notes of the outcome of the various experiments and record of special instruction as to technique may be added; and as an appendix a blank form is printed, following which as a form at the close of the work should be recorded the data ascertained in connection with the more important forms of microorganisms which have been studied in the exercises.* It has been the writer's custom to have each student carry along with the regular class work during the last two or three weeks some simple independent task, as the bacterial analysis of a water-supply, of milk, soil, or air, in which an enumeration of the bacteria found in a definite quantity of the substance examined is required, together with the identification of one or more forms of the organisms encountered, and such study of their pathogenic influence as time permits. The records of such work also may well be made after the blank form in the appendix.

* Copies of this form may be obtained from P. Blakiston's Son & Co., Philadelphia.

More than any other one measure, this practice has seemed of value in giving the student confidence in his powers of observation and reliance on his application of methods.

It has come to be a question as to how much of the general subject shall be taught in the brief course permissible in the crowded medical curriculum. In the earlier days of his teaching the writer paid more attention to the detailed characteristics of the important bacteria, especially the pathogens, than to technical work; this more because of the lack of a satisfactory system of classification of the bacteria and a chaotic condition of the general study than because he failed to appreciate the need of such system. In the more recent years, however, much advance has been made in these lines, and it is more in harmony with the purpose of education that the student should be taught in the laboratory the methods of demonstration of bacterial characters and properties, and a proper manner of investigation and observation, than that he should devote his time and attention to isolated facts which in many instances are better brought to his comprehension in connection with his work in medicine or surgery. The writer has endeavored in selection of the illustrative work to embody the more important points which clinical study demands, but in no other way than as important illustrations. Such special points cannot, of course, be too often brought forward or too much impressed, but they will probably be best remembered from their relations to clinical instruction.

There are a number of works of a systematic character adapted to bacterial determination and identification, although the perfect system of bacteriologic classification is probably far in the future; during the past year the writer has become familiar with the many excellent features of Chester's *Determinative Bacteriology*, and in the arrangement of the following pages the latter was in mind as a suitable reference book for the student in working out the identification of unknown species and for inquiry as to the important cultural characteristics of known bacteria. The admirable work of Neumann and Lehmann in its American edition is equally to be commended, particularly in its technical and descriptive portions, for class purposes.

As outlined, the entire series of exercises embodied in the following pages may be carried out in eight or nine weeks, each student working at least ten hours each week. It will be necessary, however, that the instructor inaugurate such work as will require time for its completion before the completion of preceding exercises; and it is well to have the student understand that the order of work corresponds rather with the end than with the beginning of each task.

There is, of course, nothing original in the book either in the matter or

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in the mode of presentation, both of which are more fully and more satisfactorily covered in the systematic texts upon the subject. Especial acknowledgment should be made for the use of the series of descriptive terms and the accompanying illustrations on pages 172-184, which have been taken from Chester's *Determinative Bacteriology*, 1901, with the kind permission of its author. It is only as an attempt toward fixation of systematic procedures in class work that the writer can hope to have added in the least to the advance of a subject in which so much good work has been done by a host of laborers. Should this prove true it will not be a source of regret that this scheme, imperfect and incomplete as it is, has thus been made public.

A. J. S.

GALVESTON, TEXAS, *August 19, 1902.*

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LESSONS AND LABORATORY EXERCISES IN BACTERIOLOGY.

LESSON I.

INTRODUCTION.

Plan of Work.—The student should understand that the following pages are intended to be supplementary to the general lecture instruction in bacteriology, and do not in any sense replace such instruction. Nor is the work comparable to a text-book upon the subject, being entirely too schematic and incomplete for use as such. It is intended rather that the instruction outlined in the following pages shall serve as a guide for those personal exercises and experiments which shall establish the verity of the major propositions of the systematic teachings; and shall also afford some scheme in outline for personal investigation into such problems in the subject as may be undertaken by the student in his laboratory experience. The pages are interleaved with blank sheets in order that upon the latter may be noted the results of each experiment; and in addition it is advised that these blanks be employed for the preservation of notes of all detailed instruction not included in the printed page, at least as far as technique is concerned. For the general discussions of bacteriology, the relations of the schizomycetes to allied forms of vegetation, their relations to disease phenomena, and the many and varied problems which arise in the subject generally, little or nothing may be included in a book of the scope and limitations set upon this, and reference in such lines should at once be made to the systematic texts upon the subject.

It is the purpose of the following lessons to conduct the student in some regular progression through those processes and modes of operation by which bacteria may be studied for the recognition of the individual organism and its classification in the group of the cleft fungi. The systematic and complete study of this or that form is not sought, save in a minor degree, since there must in the present crowded condition of the medical curriculum be little possibility to hope for more than a second-hand knowledge at the hands of the student of the great bulk of facts accumulated by bacteriologists in regard to individual forms of bacteria, whether pathogenic or non-pathogenic; but it is hoped that the student who has followed with punctual and earnest labor the processes herein outlined will be able, by some such analytic key as is presented, to undertake with intelligence and expectation of success the determination of such bacteriologic growths as he may encounter in his later and fuller studies. In other words, one who has pursued faithfully such work, although he may not be regarded as a bacteriologist, should be in position, by practice and further study along the same and allied lines, to become competent as such. As a matter of practice it is intended that at least a few of the more important pathogenic bacteria and such unknown organisms as may be met in material suggested for investigation by the instructor should be carried through the outlined

methods, and at the close of the book a tabulated blank is inserted, following which the preservation of the notes of observation in such work should be made.

Laboratory Cleanliness.—In the pursuit of such individual practice it is scarcely necessary to insist upon the utmost care and cleanliness on the part of each worker at all stages of the work. All appliances are to be kept in constant order and cleanliness, and whenever exposed to contamination in any manner should be subjected to thorough sterilization in the most suitable manner. Not only this, but the individual who is personally scrupulously clean is not only the most successful worker, but more certainly than the careless escapes the possible danger of infection. Suitable receptacles are provided for containing discarded material in disinfectant solutions, and such substances are later burned; and each student should bear in mind as a constant duty to see that all such matter is promptly and properly rendered harmless. Personal habits must invariably give way, in all the manipulations of the laboratory, to the precise care of surgical procedures on the living subject; and it is desirable that even in the matter of clothing similar precautions toward cleanliness and protection should obtain.

In case of accidental breakage or spilling of a tube containing a culture upon the floor, table, or elsewhere, as much as possible should be wiped up with a wet rag at once and the rag thrown into the laboratory disinfecting jar, to be burned later; and a strong disinfectant solution should be freely poured over the contaminated surface and allowed to remain for a half hour, after which it may be wiped up with a mop and the mop destroyed. Clothing thus contaminated should be boiled or baked; and the hands should be thoroughly disinfected and washed well with soap and water.

Laboratory Provisions.—Each student is furnished with a suitable work-table, with non-absorbent top, provided with water and gas connections; for general use are provided the various types of sterilizing apparatus, incubator space, serum inspissators, gas generators, material for culture media, chemical tests, staining agents, scales, inoculating apparatus, and animals for inoculation. Each student is provided with an individual locker containing the following pieces of apparatus, and can obtain by application the less frequently needed pieces for special work:

- 1 microscope with usual accessories, with high-power objective and bottle of immersion oil, and with micrometer ocular.
- 6 dozen culture test-tubes.
- 1 dozen large test-tubes for potato cultures.
- 1 large double dish for potato and plate cultures.
- 10 small double dishes (Petri dishes).
- 1 straight platinum needle with glass handle.
- 1 looped platinum needle with glass handle.
- Assorted glass rods and glass tubing.
- 1 50 c.c. pipette.
- 1 5 c.c. pipette.
- 2 glass beakers.
- 2 500 c.c. Erlenmeyer flasks.
- 1 small glass funnel.
- 1 large glass or granite funnel.
- 1 large granite pan.
- 1 granite water-bath pan (bain marie).
- 1 granite dipper, 500 c.c. capacity.
- 1 filtration bucket.
- 1 gas stove.

- 1 Bunsen burner with wing and gauze tops.
- Assorted rubber tubing.
- 1 iron tripod.
- 1 iron filter stand, three rings.
- 1 wooden test-tube rack.
- 2 wire cages for containing test-tubes.
- 1 hand brush.
- 1 bottle brush.
- 1 test-tube brush.
- 1 potato knife.
- 1 anaerobic jar and attachments.
- 1 dissecting forceps, scissors and scalpel in case
- 6 bottles with stoppers for dropping, for special reagents.
- 6 reagent bottles.
- 12 staining tubes in rack.
- 1 slide for hanging-drop culture.

Staining solutions, reagents, filter paper, wrapping paper, litmus paper, and similar test papers and solutions, cheese-cloth, etc.

In addition to the above, each student should provide himself, for personal use in the laboratory, a suitable apron or gown of washable material, soap, a number of rubber



FIG. 1.—FORM TYPES OF BACTERIA.

1. *Coccaceæ*. 2. *Bacteriaceæ*. 3. *Spirillaceæ*. 4. *Mycobacteriaceæ*. 5. *Chlamidobacteriaceæ*.

finger stalls, cover-glass forceps, glass slides and thin cover-slips, box for slides, tube of balsam dissolved in xylol, wax pencil for temporary labels on culture tubes, and several hand towels.

Bacteria belong to the vegetable kingdom of created things, constituting one group of the fungi, or non-chlorophyllous protophytes. They are also spoken of as the *schizomycetes* (sing., *schizomyces*), or *cleft fungi*, or *fission-fungi*, because among this group they are characterized by the more or less constant and common method of multiplication by direct cell division, as contrasted with sporulation, which is characteristic of the moulds, and with gemmation, which is encountered among the yeasts. While this feature is essentially a basic one, it is not absolutely constant, a few forms usually, and a number of forms under special conditions, reproducing by spore formation. As a group they are probably the lowest type of vegetables and bear many similarities to the lower protozoa (infusoria) on the one hand, and to the higher types of the fungi and algæ on the other.

They exist as unicellular organisms, isolated or in various groupings; are spherical, oval, rod-shaped, or curved in outline; and as far as known are of simple structure, in some forms only presenting as special organs flagella, by which motility is accomplished. For convenience they may be divided into the following families: *Coccaceæ* (or cocci), of

spherical shape; *bacteriaceæ* (including the non-motile bacteria and the motile bacilli and pseudomonads), unbranched and non-sheathed rods; *mycobacteriaceæ*, rod-shaped organisms approaching the hyphomycetes in forming mycelial-like threads and sometimes truly branching; *spirillaceæ*, curved rods; and *chlamidobacteriaceæ*, filamentous bacteria covered with more or less definite envelopes (Fig. 1).

For further division of these families reference may be made to the classification in Lesson IX (*vide* Chester, *Determinative Bacteriology*).

Bacteria occur widely in nature, both as free and parasitic organisms, scarcely any substance being free from their presence. In their growth *they approach the character of the protozoa* because of the absence of chlorophyll from their constitution, rendering them unfit to obtain nutrition, like the chlorophyllous plants, from inorganic substances; they are therefore to be found in greatest profusion in situations where organized food is available, and are least plentiful where such food is not obtainable for one or other reason. *The vegetable nature of bacteria is assumed* mainly from the evident close relationship to other forms of protophytes, often seen in gradation of structural and functional characters; and in the fact that at least in a number of the bacteria, if not all, cellulose or modifications of cellulose have been demonstrated as a more or less important constituent of the cell-wall. Their large proportion of proteid material, as well as many of their structural and functional features, also ally them to the lower animal life; but from a preponderance of vegetable features they are generally accepted as members of the vegetable kingdom.

Their importance to the physician lies in their frequent *parasitic* occurrence. When found growing free in dead organic matter they are spoken of as *saprophytes*. Of the parasitic forms the greater number are productive of no important influences deleterious to the well-being of the host, and may occasionally be of positive service; such are spoken of as *non-pathogenic parasitic bacteria*. However, a number are known to possess the power of inducing disease in the body of the individual invaded and are hence spoken of as *pathogenic bacteria*. It is to be recognized, however, that saprophytism and parasitism may in turn be possible in numerous instances for the same bacterium, should circumstances demand; and while an organism may ordinarily be a saprophyte, it may be capable of living as a parasite, and in the latter condition might possibly be actively pathogenic. Hence, while for the most part the essentially pathogenic bacteria demand the physician's attention, there are more reasons than that of general interest which require the study of other forms as well as of these latter.

The study of these low vegetables is known as **Bacteriology** and includes not only the general consideration of the morphology and physiology of these microorganisms, but also the technique of observations for the identification of the organism in question, and its relations to disease.

LESSON II.

OCCURRENCE OF BACTERIA IN NATURE; THEIR DESTRUCTION BY LABORATORY MEANS.

Occurrence of Bacteria in Nature.—*Bacteria are found very widely distributed in nature both as saprophytes and as parasites.*

The wide distribution of these microorganisms in nature can readily be shown by placing in material free from bacteria (sterile), but known to be suited to their nutrition, matter containing such organisms taken from a variety of sources such as may suggest themselves. It is due to this almost universal presence of these and other low forms of life that almost every organic substance, whether liquid or solid, is so apt to become destroyed by "rotting," especially if the factors of temperature and moisture of the surrounding medium be likewise favorable to the development of the germs. As illustrative examples the following experiments may be suggested and may be added to at almost any length at the will of the inquirer.

Exercise 1.—With a knife blade which has at the time been sterilized (cleansed) by careful heating in the smokeless flame of a Bunsen burner or alcohol lamp (the blade having then been allowed to cool to a convenient temperature without having been placed where it is likely to again become contaminated), some of the superficial cells of the skin of the hand are scraped from the surface and a bit of the scrapings transferred to a sterile tube of any suitable culture medium, as nutrient bouillon. The tube, resealed, is to be placed in an incubator holding a uniform temperature of from 20° C. to 37° C. for from twenty-four to seventy-two hours. After a time the presence of a living growth may be inferred by the clouding of the bouillon or by the formation upon the surface or in the mass of solid media of "colonies," appearing as drops or films of a colorless, or occasionally tinted, material. Microscopic examination will confirm the statement that such material is made up of countless organisms of some type. Note results at the close of twenty-four, forty-eight, and seventy-two hours.

Exercise 2.—In a like manner plant scrapings from the mucous membrane of the mouth. Note results at the close of twenty-four, forty-eight, and seventy-two hours.

Exercise 3.—Having sterilized the platinum loop in the naked flame and allowed it to cool, a drop of water from the tap is similarly introduced into a sterile tube of nutrient bouillon or other medium, placed into the incu-

bator, and the results noted at the close of twenty-four, forty-eight, and seventy-two hours.

Exercise 4.—In a similar manner a bit of dust from the floor or other exposed surface in the laboratory is obtained by drawing the sterilized platinum needle over it, which is then brought in contact with a prepared sterile tube of some nutrient medium, the latter placed in the incubator, and results noted at the close of twenty-four, forty-eight, and seventy-two hours.

Exercise 5.—Expose for fifteen minutes, to the atmosphere of the room, a dish preparation of gelatine or agar; close the dish and at room temperature for the former, or at incubator temperature for the latter, note at the close of twenty-four, forty-eight, and seventy-two hours the results of the probable inoculation of the medium from the air of the room.

STERILIZATION.

By this term is meant the cleansing of any substance so as to free it from such living organisms as may exist within or upon it. Just as it is necessary, in the preparation of a garden intended for the cultivation of useful vegetables, to free the soil from weeds which may interfere with the growth and excellence of the desired plants, so it is essential that all substances upon which it is intended to grow some given form of bacteria be first freed from all contaminating organisms. So, too, lest such contamination occur subsequently and more or less continuously, all containers and such other apparatus as in the necessary manipulations will come in contact with the nutrient media employed, must likewise be subjected to sterilization. Thus alone can one hope to so isolate his specimen as to make the study of its individual characteristics a possibility and to avoid a hopeless confusion and indistinguishable mixture with any number of other types of bacteria and other protophytes.

(A) To accomplish this end heat is one of the most available means at hand. It is employed in a number of different manners, each suitable for some particular substance or for some particular object in connection with the general purpose of sterilization.

1. **Flaming.**—This consists in exposing the object to be sterilized to the naked, smokeless flame of a Bunsen burner or of an alcohol lamp until it may be fairly presumed that any living matter upon its surface has been destroyed. It is usually practised for the rapid cleansing of such objects as the platinum inoculating needle, blades of knives (where the preservation of the temper of the blade is no object), glass rods, and other metallic or glass apparatus of suitable size, and such as are unlikely to be injured by the exposure. The object to be sterilized should be held in the upper, hottest, part of the flame, the time of exposure being usually one or two minutes, according to the size of the surface to be exposed to the heat, and the probable degree of contamination to be destroyed. With metallic objects, like the platinum needle, exposure is usually maintained until a distinct glow of the metal is attained. Having presumably thus burned all vestiges of life from the object, it is commonly allowed to cool to a harmless temperature before being further used, lest contact with the heated surface be destructive to such substances or organisms desired to be preserved with which the sterile object must subsequently be brought into close relation. As much care as possible should be taken to prevent contamination of the sterilized object after it has been flamed in the

moments during which it is being allowed to cool for use. Thus it is well to hold the platinum needle perpendicularly, point downward, so as to expose the least surface to bacteria settling by gravity through the surrounding air (Figs. 2 and 3), and for the same reason to hold a knife under these circumstances in a similar position or horizontally with the cutting edge downward.

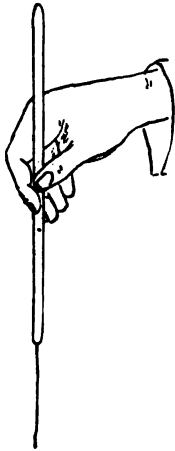


FIG. 2.—POSITION IN WHICH INOCULATING NEEDLE IS HELD WHILE COOLING.

Exercise 6.—Two tubes of sterile nutrient bouillon or other medium are provided. A platinum inoculating needle is purposely contaminated by being thrust into a tube of decomposing meat solution or other similar substance. Without further precaution it is thrust into one of the prepared tubes of sterile medium, moved gently in it for a moment and withdrawn, and the tube resealed with the usual cotton plug. The needle is now heated throughout its entire length to a cherry heat and the lower portion of the glass handle also cautiously heated; after which the needle is thrust into the second tube of sterile medium, moved about therein for a moment and withdrawn, and the tube closed. Note results at the close of twenty-four, forty-eight, and seventy-two hours so as to determine the efficiency of the flame to destroy the known bacterial contamination upon the needle.

2. Exposure to Dry Oven Heat.—The dry-air oven is employed in the sterilization of such articles as are not liable to be destroyed by drying, but which do not demand rapidity of cleansing, or are, because of their size or for other reason, not easily cleansed by exposure to the direct flame. It is usually used in sterilizing culture tubes and their cotton plugs, for the sterilization of Petri dishes, flasks, glass plates, and similar objects. The articles to be sterilized are placed in the oven, either inclosed in some suitable receptacle, as in wire cages, metal boxes, or folded in wrapping paper (as may be practised with Petri dishes, filtration bougies, glass rods, pipettes, etc.), or loose; and heat is applied in the most convenient manner. The type of oven commonly used in the laboratory is a double-walled sheet-iron box, covered with asbestos card to prevent heat loss, and provided with a suitable door. A large opening at the bottom of the outer wall permits the hot air from the flame to enter and circulate between the outer and inner walls, and a series of smaller openings at the top in the outer wall, guarded by a slide valve, allows the escape of the hot air and gas after circulation. In this way the heat is evenly distributed to the entire inclosure. A section of such hot-air oven is represented in figure 4.

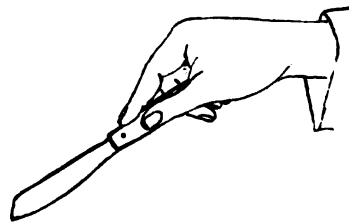


FIG. 3.—PROPER POSITION OF HOLDING KNIFE AFTER FLAMING.

While there is undoubted advantage in thus evenly distributing the heat to the different parts of the inclosed space of such an oven, it is not absolutely necessary, and the same results may be surely obtained by the employment of any available oven. The oven of any stove may be used for the purpose, a thermometer being introduced to establish certainly the presence of the requisite degree of temperature; and the same end may be attained with even as simple an apparatus as an ordinary tin cracker box (with unsoldered seams) placed upon the top of a stove or other source of heat, or in an old-fashioned Dutch oven.

For destruction of most bacteria by dry heat a temperature of 140° – 150° C. is usually regarded as essential, although many adult forms are killed by a heat much less than this. The spores of bacteria are, however, more resistant than the adult forms, and the

degree indicated may be accepted as essential for the complete destruction of all forms. For the destruction of the adult varieties, exposure of from twenty to thirty minutes to such temperature is practically always lethal; but a much higher temperature is required for many of the sporulating forms, and occasionally even prolonged exposure to 150° C. will fail to destroy some spores. In order to obviate such difficulty, Tyndall, in 1877, suggested the so-called "fractional sterilization" (or interrupted sterilization) in connection with this mode of procedure as well as in case of steam sterilization; and at present this is usually followed. Such method demands an exposure of the object to be sterilized in the oven to a temperature of 140° – 150° C., for from twenty to thirty minutes each day for three days, the intervals between the heatings being afforded so as to permit the development of any existing spores into adult bacteria and consequent loss of their powers of resistance to the heat.

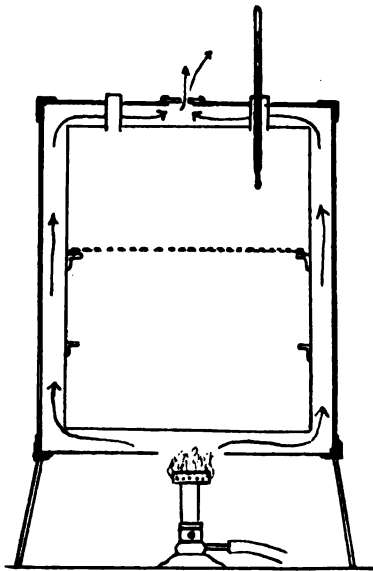


FIG. 4.—SECTIONAL VIEW OF HOT-AIR OVEN.

Exercise 7.—Select six contaminated tubes from previous exercises.

Pour out the clouded bouillon, or wash out with ordinary water the contaminated solid medium into the disinfecting jars distributed about the laboratory, taking care lest the substance be introduced into any cuts or abrasions on the hands, and at once sterilizing the hands by washing for at least five minutes in a one in two thousand solution of bichloride of mercury. Into the first of these tubes, *without further preparation*, introduce a few cubic centimeters of sterile bouillon, and close with sterile cotton plug, as usual. Heat a second tube in the hot-air oven, in which a temperature of 150° C. has been attained, for *five* minutes; and in the same manner there are to be added a few cubic centimeters of sterile bouillon, and the tube

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again closed with its cotton plug. A third tube is heated similarly for *ten* minutes, bouillon added, and the tube closed. A fourth tube is heated for *twenty* minutes, bouillon added, and the tube closed. The fifth tube is heated for *thirty* minutes and similarly treated; and the last tube is heated for *twenty minutes each day for three days*, to the same temperature, after which sterile bouillon is added and the tube closed and placed in the incubator, which should also have been done with the former tubes. The results of such heating in each case should be noted and compared with the results obtained in the unclean and unheated tube, at the close of twenty-four, forty-eight, and seventy-two hours.

Exercise 8.—A tube containing some marked growth is selected. Inoculation is performed from this material to a fresh tube, the latter closed and placed in the incubator. The original tube is then subjected to dry heat for *five* minutes, *ten* minutes, *fifteen* minutes, *twenty* minutes, *twenty-five* minutes, and *thirty* minutes. At the close of each interval a fresh tube of sterile medium is inoculated from the original tube, and after being closed with its sterile cotton plug, is placed in the incubator, and results are noted as usual, at the close of each twenty-four hours, for three days. *That tube in which growth first fails to occur probably corresponds with the lethal exposure to the temperature to which the tube was heated (vide Thermal Death-point).*

3. Boiling.—The temperature of boiling water (100° C.) is easily destructive of bacterial life if long enough continued or if the conditions for penetration of the heat are favorable. This ease of penetration is present best when the bacteria to be destroyed are suspended in the fluid (rather than when inclosed in fabric or other solid) which is being boiled, the hot liquid coming into close contact with the organisms in every part and on every side. As a rule it may be said that sterilization by boiling is quite as efficient as by exposure to a temperature of 150° C. of dry heat. Boiling is particularly useful in the sterilization of articles likely to be destroyed by dry heat, as clothing, non-coagulable nutrient fluids, glassware which may be cracked by any irregularities in the application of the oven heat, etc.

Exercise 9.—Boil water for *five*, *ten*, *fifteen*, and *twenty* minutes, and at the close of each interval add several drops to a tube of sterile medium; close, place in incubator, and at intervals of twenty-four hours for three days note the results obtained. Compare with a tube inoculated with water not previously boiled.

4. Steam Sterilization.—Steam heat is commonly used for the sterilization of such substances as are likely to be injured by heating in the dry-air oven, and which cannot conveniently be boiled or sterilized by chemical or mechanical means. It is most frequently used in connection with the various culture media, and in practical medicine and surgery in the sterilization of dressings for wounds, of clothing and similar substances. The temperature of steam unconfined by the lid of the steam chamber is approximately 85°

C.; that of partially confined steam (as in any of the common steam sterilizers, where the lid of the receptacle is closed, but not tightly clamped, and where perhaps there is in addition some opening in the lid also serving as a means of moderate escape of the vapor) is about 100°C . or that of the boiling water; while the temperature of steam confined under pressure, as in the specially constructed steam boilers known as *steam digesters* or *autoclaves*, may be raised considerably above that of boiling water. The exact degree in the latter case varies with the degree of confinement, whether one or more atmospheric pressures above normal, and with the amount of water in the boiler (the less water after development of the steam, the higher the temperature for a given application of heat).

Steam as a sterilizing agent is of value because of its penetrative power as compared with dry heat, the evenness of its contact, and steadiness of its action.



FIG. 5.—Koch STEAM STERILIZER.

Exposure to steam at 100°C . may be reckoned as of equal efficacy for sterilization as dry heat at 150°C .; and ordinarily it is the custom to allow the same period of exposure as that employed in the use of dry heat, twenty to thirty minutes, at intervals of twenty-four hours, upon three successive days. If the substance to be sterilized is not liable to injury upon prolonged exposure, it may be left in the steam at 100°C . for an hour with a fair certainty that all adult bacteria and spores will at the end of that time be destroyed. For rapid and complete sterilization, however, exposure to high temperature in steam under pressure in an autoclave is usually practised, a temperature of 120°C . and pressure of thirty pounds to the square inch being usually employed, an exposure of from fifteen to twenty minutes being generally lethal to both adult bacteria and their spores.

(a) In ordinary steam sterilization some one or other form of steam boiler is made use of, in which is attained a temperature of 100°C ., or under special arrangement somewhat less.

Perhaps the simplest type, and that most convenient for a variety of purposes, is the Koch pattern (Fig. 5), consisting of a rather tall, heavily tinned sheet-iron boiler, with copper bottom, arranged upon an ordinary iron tripod or upon an inclosed iron stand, covered with asbestos felt to prevent the rapid radiation of heat, and fitted with a convenient water gauge and a moderately close fitting cover in which a suitable perforation is often provided for the accommodation of a thermometer. In the interior above the water-level is arranged a false bottom of perforated metal. When in use, water is put in the boiler so as to cover the bottom for four or five inches, but not enough to reach the false bottom; heat is applied by a convenient burner and the water brought to the boiling-point. The articles to be sterilized are placed in the steam chamber, resting upon the false bottom, and the cover applied. There is no need of watching the temperature, and there is therefore no occasion for a thermometer, since the temperature cannot rise above 100°C . with no

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more confinement than afforded by the ordinarily fitted lid. If the objects subjected to the steam-bath are not liable to injury from long exposure, the sterilization may be accomplished by a single steaming for an hour; but ordinarily, to prevent injury and for greater certainty of result, the interrupted method of Tyndall is followed, the articles, after subjection to heat for fifteen or twenty minutes, being removed from the bath, and the process repeated twice at intervals of twenty-four hours.

(b) Another form of steam sterilizer often employed is the Arnold sterilizer (Fig. 6), which possesses the advantage of quick generation of steam, an advantage often much appreciated in laboratory work. As shown in the diagram in section, this apparatus consists of a steam chamber placed over a thin plate boiler, and communicating with the latter by an open cylinder through which the steam generated in the boiler

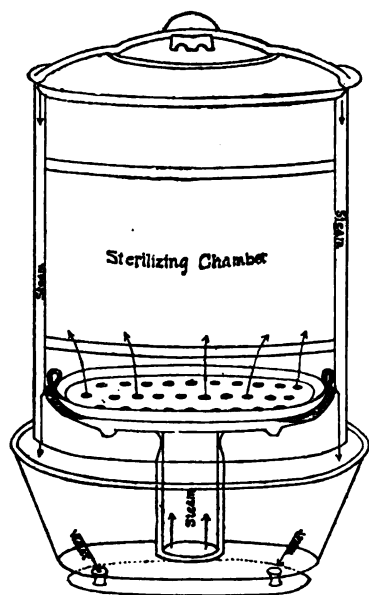


FIG. 6.—DIAGRAM OF ARNOLD STEAM STERILIZER.

is conducted into the chamber. A water reservoir surrounds this cylinder and communicates with the boiler beneath by means of several small feed tubes for the supply of water as it is vaporized. In the bottom of the steam chamber a perforated false bottom is placed for the accommodation of the articles to be subjected to sterilization. A light cover is applied to the steam chamber, loosely enough to allow the moderate escape of excess of steam. A hood of metal covers the chamber, in the interior of which the escaping steam is caught and condensed, running down its sides and dropping back into the reservoir, to be used over again. In use, the reservoir should have water poured into it until it stands about half or three-quarters of an inch in depth over the bottom, the boiler being thus also filled. A moderate-sized burner is placed near the middle of the bottom, and in a few minutes a clicking sound is heard, indicating the boiling of the water. The articles to be sterilized are now placed in the interior, the cover and hood adjusted, and after a few minutes more a sterilizing heat has been attained. Exposure in this apparatus should be the same as in the case of any other form

of apparatus. After proper exposure, if one is sterilizing such substances as fabric, or tubes plugged with cotton stoppers, it is well in this, as in other forms of steam sterilizers, to give free vent to the steam by raising the cover somewhat, lest the contained steam, in cooling, condense upon the material and render it wet. A caution should be observed in the employment of the Arnold sterilizer as to the heat applied. Should the flame be fierce and the temperature employed very high, it is not safe to depend upon the maintenance of the water-level in the reservoir; and should the water disappear, the intricate joints of the apparatus may have the solder melted, especially about the small feed tubes, the damage costing considerable inconvenience and expense in repair. If used with ordinary care there is little danger of such accidents, but with a large flame, as from a gas stove, the whole apparatus is apt to become overheated, and the condensa-

tion of the steam in the hood does not take place with certainty, the level being quickly lost or all the water evaporated, after which the damage is done. With a proper flame, the reservoir once filled, the apparatus requires comparatively little attention; but unless students are careful the appliance is likely to be more costly than the more simple types.

In private work requiring steam sterilization it should be remembered that much simpler apparatus than either of the forms mentioned may be extemporized which will accomplish all that is requisite, the expense attendant upon these set forms being entirely unnecessary. A cheap tin bucket, preferably of a tall shape, should be obtained,

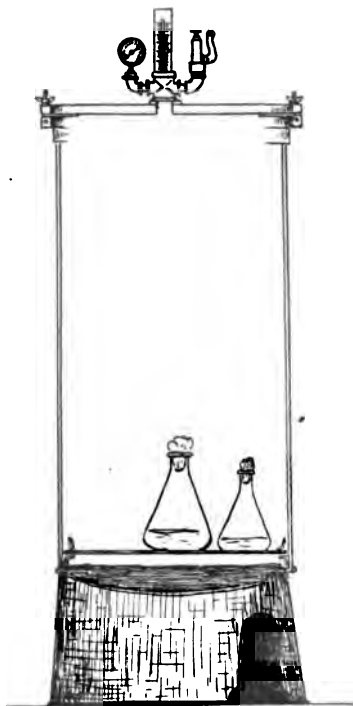


FIG. 7.—SECTIONAL VIEW OF AUTOCLAVE.

several pieces of stone or brick placed in the bottom, and a piece of metal, perforated in any convenient manner, arranged upon these as supports, thus forming a raised false bottom. Water is put into the bucket nearly to the level of the false bottom, the articles to be sterilized introduced, and the cover adjusted and heat applied. Some little additional care is requisite to see that the water is not evaporated before completion of the process; but otherwise such a crude apparatus presents no disadvantages and will serve the purposes demanded as well as apparatus costing a number of dollars.

(c) For rapid and thorough sterilization an *autoclave* or steam digester is commonly employed. In principle it is merely a heavy metallic boiler, capable of withstanding considerable steam pressure, fitted with an equally strong cover with tight fittings and clamps for close application to the boiler. There is usually a steam gauge fitted into the cover, and a closed tube sunk in the cover for the reception of a thermometer; a safety valve, set to whatever pressure is desired, is provided in the cover or elsewhere; and generally, for convenience, a small pet valve, closed by a screw plug, is also arranged. In order to conserve the heat of the flame the boiler is usually set upon a closed stand of heavy sheet iron, the inclosed flame not being deviated by draughts, and little heat lost by

radiation. The apparatus can readily be understood by reference to the diagram of a section (Fig. 7). In the interior there is a false bottom or gauze bucket or other container for the reception of such articles as are to be subjected to the process. There should be but little water put into the boiler (the bottom being covered with about an inch of water), and a strong flame applied. At first the cover should be but loosely applied and the pet valve opened. When steam begins to escape, the articles to be sterilized are to be placed in the boiler, the cover clamped in position, the pet valve closed, and the time noted. Usually, an exposure of fifteen minutes is employed, after the temperature has risen to 120° C., the pressure at the same time registered by the steam gauge being about thirty pounds to the square inch. The heat is then with-

drawn, and the temperature allowed to fall to somewhat less than 100° C., and the pressure to normal, before the cover is removed and the sterilized materials taken from the interior. Should one attempt to open the cover without having allowed equalization of pressure to have taken place, fluids in the interior, as culture media in flasks or tubes, are liable to bubble violently, wet the stoppers, or the escaping gases may force them from the necks of the flasks or tubes; and the operator, if careless, is liable to accidental scalding from the rush of steam from the apparatus. One such exposure is easily equal to the three exposures in the ordinary method of operation, but it should be recalled that the high temperature may be unsuited to some materials.

Exercise 10.—Repeat exercise 8 in duplicate, employing, instead of dry heat, for one set of tubes the ordinary fractional sterilization in steam without pressure, and for a second set sterilization in the autoclave, temperature 120° C. and a pressure of thirty pounds. In the first set allow one infected tube to be exposed to the full process, one to but two exposures, one to a single exposure of twenty minutes, one to a single exposure of one hour. In the second set let one tube be exposed to the full twenty minutes' heating, one to steam for fifteen minutes, one ten minutes, and one five minutes. Place all tubes in the incubator and note results at the close of twenty-four, forty-eight, and seventy-two hours. The tube in each case with least exposure showing no occurrence of growth marks the lethal exposure of the given germ to the mode of procedure employed.

In determination of the *thermal death-point* of a given microorganism, material devoid of spores is selected (this determined by microscopic examination or by the success of inoculations from cultures which have been exposed to a temperature of 80° C. for ten minutes). This material may be either diffused in a sterile fluid in tubes or smeared dry on sterile glass slides; each specimen is then placed in one of a series of ovens the temperature of which ranges several degrees apart through a number of degrees, as 50°, 52°, 54°, 56°, 58°, and 60° C. At intervals inoculations are made from each specimen. The shortest exposure to the lowest degree of heat after which such inoculations fail of growth represents the temperature lethal to the bacterium. In the record note should be made of whether the organism was in moist or dry condition, as well as the degree of temperature and the duration of exposure.

Exercise 11.—In order to observe the relative value of steaming without pressure and under pressure, some infected material, as a fresh surgical dressing from some suppurating wound or bits of sterile threads dipped in a bouillon culture of some known organism, as the colon bacillus, is carefully wrapped in a known number of thicknesses of a towel. One such preparation is to be placed in the autoclave and subjected to the usual steam exposure; the other is placed in the ordinary steam sterilizer for the same period. As soon as removed, inoculations are to be made from each of these preparations to fresh tubes of nutrient medium, incubated, and the results noted as usual at the close of twenty-four, forty-eight, and seventy-

two hours. Should both prove sterile, the experiment is to be repeated with briefer period of exposure.

5. Pasteurization.—This term is applied to a partial sterilization accomplished by exposure to a comparatively low temperature (60° – 90° C.) for a period of time ranging, under varying circumstances, from fifteen minutes to an hour. While in the briefer exposures to the lower ranges of temperature the sterilization is usually but partial, with longer exposures to temperatures somewhat less than boiling heat, especially steam heat, and particularly when the procedure is repeated from time to time, the destruction of bacteria present may be complete. In its ordinary sense the process consists of steaming the material in hand at a temperature of 60° C. or over for fifteen to thirty minutes; it is especially employed for the preservation of milk, this exposure being sufficient to destroy at least most of the pathogenic and souring bacteria which have been present in the sample, but insufficient to cause any material physical change in the milk. The procedure in this connection was instituted to avoid the changes which occur in milk upon complete sterilization at higher temperatures and which render it objectionable for infant feeding. Essentially the same process, however, had long been employed in the preparation of clear solidified blood-serum, although in the latter case the higher temperatures (ranging from 70° to 85° C., and even higher, but not to the boiling-point), longer exposures, and repetition of the method (one hour daily for a week) had been practised. Originally, in the preparation of blood-serum an ordinary steam-bath was used; but for years dry heat in a chamber inclosed in a water-bath (*serum inspissator*) has been employed. One may therefore look upon Pasteurization as merely the application of either steam or dry heat at a low temperature for partial or complete sterilization, such temperature more or less completely destroying contained bacteria without causing material alteration in the constitution of the substance handled.

A number of special devices are used for Pasteurization, especially in the preparation of milk for infants' food; but the same result may be accomplished by heating the milk or other substance in freely streaming steam (temperature approximately 80 – 85° C.), or in a water-bath, for fifteen to twenty minutes.

Exercise 12.—Into each of three sterile test-tubes a few cubic centimeters of sweet milk are introduced and the tubes closed with sterile cotton plugs. One is at once placed in the incubator. The second is Pasteurized in streaming steam for twenty minutes. With a sterile pipette the student should withdraw a few drops from the latter to observe the preservation of the normal taste of the sample, and should note also the preservation of the normal color of the specimen. The third tube is introduced into the autoclave and heated to 120° C. for fifteen minutes. On withdrawal note should be made of the yellowish or brownish alteration in color usually produced, and with a sterile pipette a few drops are to be withdrawn in order to observe the change in taste also caused by the heat exposure. The sterilized and Pasteurized tubes are now to be placed in the incubator with the first tube and daily note, for at least three days, made of each as to coagulation, color, reaction to litmus, visible growth, etc., to determine the differences in effect of the two processes upon the preservation of the milk.

(B) **Sterilization by Filtration.**—The principle of separation of solid admixtures from fluid media by means of filtration through porous substances has several applications in bacteriology in the matter of sterilization.

(a) While bacteria are, of course, of much smaller size than the spaces in ordinary filter paper or the meshes of a cotton plug or fabric, without the force of currents of liquid to carry them they are unable to pass, save by continuous growth, through the intricate spaces of a moderately thick layer of the paper or cotton; and plugs of cotton or several thicknesses of paper or fabric are therefore commonly utilized, after thorough sterilization, as a means of protection to the interior of flasks, tubes, etc., from the entrance of bacteria from the surrounding air, although permitting fairly free ingress and egress to the atmosphere itself. Moreover, if such material be kept dry, and free from soiling with any of the nutrient substances contained in the flasks or tubes, there is no opportunity for the penetration by growth of any organisms settling upon their exposed portions, and the protection from external contamination thus afforded is practically complete.

Exercise 13.—From a small glass tube blow a bulb as exhibited in figure 8. Plug each end with a bit of cotton and sterilize in the usual manner.

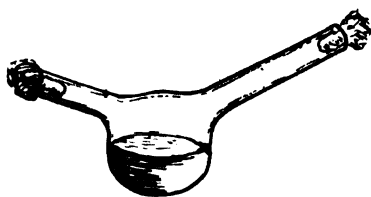


FIG. 8.

Introduce into the bulb a small amount of melted agar or gelatine and again sterilize the whole. Now attach the apparatus to a suction pump and for several minutes draw through the tube and over the medium contained in the bulb air which has been filtered through the cotton plug in the exposed orifice of the tube. Repeat the same with a

second tube similarly arranged, but not having a cotton plug in the end of the tube, the air drawn through the tube being unfiltered. Place both tubes in the incubator and at the end of the first, second, and third day note the presence or absence of growth in each.

(b) Filtration through various porous substances has commonly been used in the crude purification of water and other liquids; and the same principle is employed in the laboratory for sterilization of liquids from bacterial contamination when the ordinary methods of heating or chemical purification are not to be permitted because of the important physical changes liable to be produced in the liquids subjected to such processes. Filtration through paper, cotton, or other coarsely porous substances is, however, of little or no value, the bacteria in the fluids being readily conveyed with the currents of the liquid through the spaces of the filter; and specially prepared filters made of unglazed porcelain are commonly employed for the purpose. The Pasteur-Chamberland filter, widely used for the filtration of water for drinking, is an excellent type. Among various models, one of the most simple and efficient is that of Kitasato, a view of which is shown in the accompanying diagram (Fig. 9). Such porcelain filters may be utilized in the sterilization of various liquid culture media, of water, or in the separation of bacteria from infected media when it is desired to obtain the various chemical products of bacterial activity free from the bacteria themselves. When used, inasmuch as

the liquids, particularly solutions of organic matter, pass but slowly through the rather compact porcelain, it is commonly necessary to attach to the receiving flask some form of vacuum pump, an ordinary water pump connected with a neighboring faucet being usually used. Care should be exercised to have the connection between the pump and the receiver of heavy, non-collapsible tubing and so arranged that there is no danger of reversal of the flow of water so as to enter the receiver. Before setting up such filtration apparatus it is essential that the various parts be sterilized. Those parts made of glass, as the heavy receiving flask and funnel, having been well washed, are to be plugged with cotton and baked in the ordinary manner or sterilized in the autoclave. The rubber connection and the perforated rubber stopper should be soaked in a solution of some suitable disinfectant (as 1 : 1000 solution of mercuric chloride, or a three or four per cent.



FIG. 9.—KITASATO FILTER.

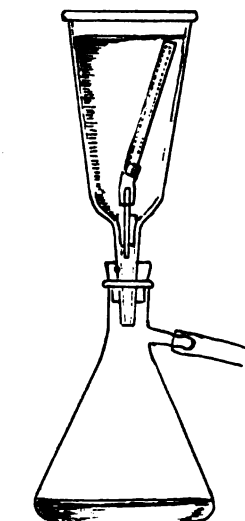


FIG. 10.—FILTER REVERSED IN A PERCOLATOR FILLED WITH WATER IN ORDER TO WASH BACK SOLID PARTICLES LODGED IN ITS WALL.

solution of carbolic acid) and well rinsed in sterile water. The porcelain bougie should have been wrapped in paper, the open end of the bougie being marked on the outside, and carefully sterilized in the autoclave. All of the parts having received such attention, the funnel is adjusted in the perforated rubber stopper, the connecting rubber tube fitted to the lower end of the funnel; and the open end of the bougie being exposed from its paper cover is adjusted in the lower end of the rubber connecting tube, which is bound to the latter and to the lower end of the funnel by several ties of sterile copper wire. The bougie is then freed from its wrapping paper and placed in the receiving flask and the rubber stopper tightly fixed in place. The pump connection is then made with the side tube of the receiving flask, the fluid to be sterilized introduced into the funnel, and the pump cautiously started. At best filtration is a slow process, and if proceeding at all satisfactorily should not be interfered with, since the least extra force of current

through the porcelain may be capable of conveying some of the bacteria through the filter. Moreover, after a time, with any degree of forced filtration there is danger that the smaller forms of bacteria will slowly penetrate through the porcelain. For this reason the value of this form of sterilization is limited and to be recommended only when other methods are not available for the purpose desired. In the selection of the filter care should be exercised that the porcelain is of even and compact composition, and of considerable thickness where the amount of fluid to be filtered is at all large. After a filter has been used its further value for sterilization is often doubtful, for several reasons. It may, it is true, be cleansed by reversing its position in the apparatus (Fig. 10) and drawing through it a large amount of clean water, thus washing back the microbes and other solid particles lodged in its interstices; after which it is to be resterilized in the usual manner in the autoclave. Such cleansing is, however, in many instances of little practical efficacy, and the permeability of the filter in consequence diminished even if the foreign particles are of no vitality after exposure to the heat of the autoclave.

Exercise 14.—Having prepared and set up a Kitasato or other filter as above outlined, filter some tainted meat solution into the receiving flask. Let each student then inoculate a fresh tube of sterile nutrient medium from the filtrate, place the inoculated tube in the incubator, and note results as usual at the end of the first, second, and third days in order to determine the efficiency of the process as a means of sterilization.

Exercise 15.—Repeat the above exercise, employing some large, known bacterium, as the *Bacillus subtilis* in a hay infusion, as the material for inoculation; and in the same way note results after inoculation of the sterile nutrient medium with such material.

(C) **Sterilization by Means of Chemical Agents.**—Various chemicals in solution or in vapor are commonly employed for the purpose of bacterial destruction or for retardation of their growth, the terms *disinfectant* and *germicide* being used to indicate such substances as are capable of destroying microbic life, and *antiseptic* to indicate such as are merely capable of restraining the growth or other functions of bacteria without actually killing them. The confidence which was formerly reposed in many of the so-called disinfectants has in recent times been considerably disturbed by the discovery that in many instances the bacteria apparently killed are really living, and can by proper procedures be caused to resume their vital activities. It was formerly supposed that such substances as *mercuric chloride*, which may be taken as a type of the disinfectants, were directly lethal by mere contact with the germ, the chemical substance itself not being modified by such contact. Recent studies, however, indicate that true chemical compounds are formed from organic materials and the metallic base of the salt, spoken of collectively as *mercurial albuminates*, the haloid element being evolved in free state and itself further combining. Such destruction of the disinfectant and combination of its elements must, of course, render inert for further use in sterilization just such proportion of the salt as has entered into the chemical change; and the albuminate, thus formed in greater or less amount, acts as a protective covering around the individual germs and upon the exterior of any mass which should be penetrated by the disinfectant solution. This coating may effectively protect the individual germs against further activity of the disinfectant, and, although mechanically preventing in greater or less measure the manifestation of their vital phenomena, may permit the maintenance of

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life. It has been shown that solution of such covering from the exterior of the bacteria, by means of some chemical solvent, as a weak solution of ammonium sulphide, may be followed by resumption of the life activities of the germs if they be transferred to a favorable surrounding. Considerable revision of opinion, particularly in connection with the inorganic disinfectants, as to their relative sterilizing value has thus been enforced. At present it is believed that the organic disinfectants, as *carbolic acid*, and the haloid elements, *chlorine*, *bromine*, and *iodine*, are less liable to give rise to such organic combinations than the formerly esteemed metallic salts, as those of mercury, silver, copper, and iron; and the former are therefore more generally commended than formerly for the purposes of sterilization. The objection mentioned concerning bichloride of mercury obtains in varying degree with the other disinfectant salts of mercury and salts of other metallic bases; but it should be recalled that this objection may be largely obviated, if in their use in disinfection one remembers that the process is essentially a quantitative one and employs a *considerable excess of the disinfectant solution* and allows a long action period for thorough contact of the chemical with the bacteria in the mass or liquid sought to be sterilized. In this connection it may be insisted upon as a second and absolute requisite in the practice of disinfection by chemical means, that there must be *intimacy of contact* between the disinfecting substance and the bacteria to be destroyed, and that such contact *must be maintained* for at least a known minimum of time. There may thus be formulated as a rule for chemical disinfection that in all such usage there should be employed a free excess of the disinfectant, that it should be caused to penetrate and diffuse through the mass or liquid subjected to its action so as to be brought in intimate contact with the contained bacteria, and that it should be allowed a sufficient period of time in which to accomplish the destruction of the bacteria.

In the use of these chemical agents in liquid form, they are almost invariably found more efficient if moderately heated (40° C., or above). They are not comparable to the action of heat and are to be used only when every form of heating is unavailable for one or other reason. Among the substances for the sterilization of which the chemical disinfectants are usually selected, may be mentioned such pieces of glassware and laboratory apparatus which on account of size cannot be introduced into the sterilizing ovens or steam chambers, material like rubber goods, which would be injured in the heat, refuse of various kinds, as the rejected contents of infected tubes (which should after collection in such disinfectant solution be burned to render its destruction certain), the clothing and dejecta of patients affected by infectious diseases, and a variety of similar substances.

Disinfectant Solutions.—Of the various disinfectant solutions, in spite of the objection above mentioned, that of *mercuric chloride* (1:1000 or 1:2000) has been and continues to be the most universally employed. Efforts to prevent the formation of the organic compounds of mercury have been made by the addition of small proportions of such materials as hydrochloric acid, sodium or ammonium chloride, or tartaric acid to the solution; but it is doubtful whether any of these, save the last, serves any efficient purpose or indeed does not hasten the active decomposition of the mercurial salt. If used in free excess over the amount of material subjected to it for disinfection, the following stock solution offers a convenient and efficient medium for laboratory use:

Mercuric chloride,	200
Tartaric acid,	500
Distilled water to	2000

Ten cubic centimeters of the above to a liter of water make a solution of 1:1000

strength. In use this solution, as all others of the chemical disinfectant solutions, should be allowed, for action, from half an hour to any longer period which can profitably be permitted, and should always be used in great excess of volume. All waste and useless materials thus sterilized should be subsequently burned to obviate the danger of failure of sterilization; and any articles to be further used should be well rinsed in sterile water before being employed.

Among the other salts of mercury the *iodide* and the *nitrate*, in practically the same proportions as the chloride, may also be employed in disinfection.

Carbolic acid is probably superior to any of the metallic salts in the certainty of its disinfectant action, but is slower and should be kept in contact with the objects to be sterilized for periods ranging from an hour to twenty-four hours in length. It is usually used in solutions of two and a half to five per cent. strength. The addition of an equal proportion of hydrochloric acid to the solution materially increases its activity, and forms one of the most valuable laboratory disinfectants. It also forms an inert compound with organic substances with which it comes in contact, but to a considerably less degree than the metallic salts, to which it is therefore superior.

Formaldehyde possesses valuable germicidal action if used in solutions of from ten parts of the commercial article (a forty per cent. watery solution of formaldehyde gas) to 1000 parts of water, to forty parts to 1000 of water. It has little penetrative power into albuminous substances, however, and must be granted considerable time for its complete action. Its odor and irritant properties prevent its general use.

Chlorinated lime is one of the most valuable disinfectants, in fresh solution, which we possess, depending upon the free chlorine evolved by its decomposition. It should be used in solutions of from one to three or four per cent. strength, made from the fresh commercial substance as required for use, as it readily decomposes. Articles to be sterilized should be kept in contact with an excess of such solution for at least an hour; and, as is always required, should be subsequently rinsed in sterile water if intended for further use. It is an excellent disinfectant in the sick-room for sterilization of clothing or dejecta, and may be profitably used in the laboratory waste jars for preliminary disinfection of the matter therein collected for combustion. *Ordinary lime* in strong (twenty per cent.) solution (milk of lime or whitewash) is an excellent crude disinfectant, especially useful in the purification of dejecta from subjects of such infections as typhoid fever or cholera.

Copperas (ferrous sulphate), commonly used as a disinfectant in urinals and cess-pools, is of comparatively feeble germicidal value, but possesses considerable power as a deodorant. *Potassium permanganate*, frequently employed in one of the steps of surgical disinfection, is of little value, even in comparatively strong solution.

For discussion of the numerous other agents employed in disinfectant solution, reference should be made to the various text-books upon bacteriology.

Antiseptic Solutions.—Of the antiseptics, borax, boracic acid, iodoform (from the iodine set free from its gradual decomposition), many of the essential oils (as of cinnamon, cloves, thyme, mint, and other plants), as well as weak solutions of the disinfectants, may be mentioned. Oil of mustard is both antiseptic and deodorant, but is too irritant for common use; the dry mustard meal is often used to remove odors from the hands after performance of post-mortem operations and similar work. The retardation of development of any germ by such substances is not the only phenomenon of antiseptics. In addition to or in place of prevention of growth, the presence of such substances may modify the mode of multiplication (perhaps inducing sporulation), the physical characters of the resultant colonies (as in shape or color), or the pathogenic virulence or other

biologic properties of the bacteria studied. However, the modification in the rate of development is generally taken as an index of the effect of all such antiseptic influence.

In the production of antiseptics it is supposed that the antiseptic substance is continuous in its presence and proportion in the medium, although upon the least reflection this must be matter of question. Were such substance able to prevent or modify growth by its mere presence, its continuous presence thus being unmodified must result in the indefinite prevention of development and the eventual natural disappearance of the germs. It is probable that the activity of antiseptics must be regarded, just as that of the disinfectants, not as a catalytic one, but as a process of quantitative changes; and that retardation of development persists only until a certain amount of the antiseptic present has been rendered inert by combination or dissociation. This accomplished, such of the bacteria present as are not actually destroyed are no longer interfered with, but develop in their original and usual manner and with their natural characteristics. Further study is required for the establishment of the truth of a number of problems connected with the subject.

Determination of Disinfectant Values.—The greatest dilution of a given substance placed in excess in contact with a given bacterium for the shortest action period (time of exposure) causing the destruction of that organism is spoken of as the *disinfectant value* of that substance for the germ employed in the test. Thus, one may say of bichloride of mercury that it is disinfectant in 1:1000 solution in five minutes for the anthrax organism in its vegetative form, although its spores may under favorable conditions resist the same solution for several days. From what has already been said, however, it must be clear that such statements are true only in variable degree, according to the medium in which the organisms to be destroyed may be contained. A much shorter period of exposure and a greater dilution of the disinfectant will accomplish destruction of bacteria suspended in an albumin-free liquid than could be brought about in the same time by even stronger solutions of the chemical acting upon the same organisms embedded in a highly albuminous mass or in a strongly albuminous liquid. In determining the germicidal value (or the antiseptic value as well) of any substance, therefore, for the sake of uniformity the germs exposed to disinfection should invariably be suspended in the same type of surrounding medium, a one per cent. peptone solution being commonly selected for the purpose.

For the determination of the disinfectant value of any substance there are a number of methods in vogue, one of the most simple and satisfactory of which is that devised by Sternberg, the principles of which are followed in these directions: Several tubes, each containing a known quantity of virulent culture of a given organism in peptone solution, are prepared. To each a definite amount of the disinfectant to be tested is added, so as to make a series of dilutions of the germicide in known proportions. Each is agitated so as to thoroughly diffuse the disinfectant, and at regular intervals inoculations are made from each into fresh tubes of the nutrient material, incubated, and the results noted as usual at the close of each day for three days. *That tube representing the least proportion of the disinfectant, acting for the shortest period, in which no growth follows, corresponds to the disinfectant value of the substance for the selected microorganism.* If it be objected that the small amount of the disinfectant transferred in the inoculation of the fresh tubes has exercised in these tubes a restrictive influence, this may be estimated and corrected by a control experiment, by taking the same amount (a loopful) of the disinfectant substance in the same degree of dilution in peptone solution as that represented by the original tube (that corresponding to the accepted germicidal value of the disinfectant), transferring it to a second tube of the

same medium, diffusing it by gentle agitation, and then planting therein from a virulent culture of the germ employed in the test, so as to note whether such proportion exercises any destructive or restraining influence.

Exercise 16.—Prepare four tubes of one per cent. peptone solution, each containing four cubic centimeters; inoculate each with the *colon bacillus* and incubate for twenty-four hours, protecting the contents of the tubes against evaporation by a rubber cap or rubber stopper over the usual cotton plug. Having the following day made certain of the success of the infection of each tube, add to the first one cubic centimeter of a five per cent. solution of carbolic acid (*thus making a one per cent. dilution of the acid in this tube*); to the second, one cubic centimeter of a ten per cent. solution (*two per cent. solution in the tube*); to the third, one cubic centimeter of a fifteen per cent. solution (*a three per cent. solution in tube*); and to the fourth, one cubic centimeter of a twenty per cent. solution (*four per cent. in the tube*). Diffuse the disinfectant immediately in each by gentle agitation. After appropriate intervals (*five, ten, twenty, thirty, forty, fifty, and sixty minutes*) transfer with the platinum loop one loopful from each tube to a fresh tube of the peptone solution. Each tube, properly marked, is incubated, and, at the usual intervals of one, two, and three days, observations are made and recorded. What tubes permit growth? What tubes show no development? That tube planted from the greatest dilution of the carbolic acid, having had the shortest action period, which shows no growth, may be taken as representing the disinfectant value of this substance for the *colon bacillus*. To verify the result, make a preparation of the same degree of dilution of the acid in a sterile tube of the peptone solution, and inoculate it with a loopful of a virulent culture of the organism. If growth readily follows, it may be inferred that no material influence was exercised by the small amount of carbolic acid transferred in the test experiment; if growth occurs but slowly, retardation only may have been accomplished, and in such event the contents of the tube accepted as indicating the disinfecting power of the acid in the test experiment should be diluted freely with sterile peptone solution, further incubated, and observed. If no growth should follow, however, it may be accepted for practical purposes that actual disinfection, and not mere retardation (antiseptis), was accomplished.

Determination of Antiseptic Values.—It should be recalled here, as in the case of disinfection, that the antiseptic value must vary for every substance according to the microorganism subjected to its influence, and probably according to the character of the surrounding medium as well. The method for determination is a simple one, consisting of the addition of known amounts of the material under investigation to known amounts of sterile nutrient medium (one per cent. peptone solution), each tube thus prepared

being then inoculated with the test bacteria. That tube containing the least amount of the antiseptic in which growth fails to appear represents the antiseptic value of that substance in relation to the particular bacterium used in the test. However, in such tube it should be possible, upon the addition of an excess of the peptone solution (thus further diluting the antiseptic), to obtain the characteristic development of the organism.

Exercise 17.—Four tubes, each containing four cubic centimeters of a one per cent. solution of peptone, are prepared. To the first is added one cubic centimeter of a two per cent. solution of borax (*making a 1:250 solution of the borax in the tube*); to the second, one cubic centimeter of a one per cent. solution of borax (*1:500 solution in tube*); to the third, one cubic centimeter of a half per cent. solution of the borax (*1:1000 solution in tube*); to the fourth, one cubic centimeter of a quarter per cent. solution of the borax (*1:2000 solution in the tube*). Inoculate each tube with a virulent culture of the *colon bacillus*, and incubate for three days. As a control, a tube of the same medium is to be inoculated at the same time with the germ, no borax having been introduced. Note the appearances presented by each tube at the close of each twenty-four hours. At the end of the third day select the tube which fails to show any bacterial contamination, having the least proportion of borax present. Dilute the contents with an equal amount of sterile peptone solution and again incubate. Growth should now follow. Should it fail it is to be presumed that the proportion of borax contained therein has acted rather as a disinfectant; and the tube containing the next lower proportion of the borax should be similarly treated to determine the persistence of vitality of the bacilli. The first tube of the series which thus presents growth after dilution with more of the culture medium, but which before dilution failed to exhibit development of the organisms which had been transplanted therein, represents the antiseptic value of borax for the *colon bacillus*.

Exercise 18.—To demonstrate the possible failure of a disinfectant because of the formation of a protective layer of organic compounds of the disinfectant with the nutrient medium or with the outer portion of the bacterial wall, thus encasing the bacteria or their spores and preventing proper contact between the disinfectant and the microorganismal body, the following exercise should be practised: Suspend for several hours five bits of sterilized silk or cotton thread, each about two inches in length, in a bouillon culture of the hay bacillus (*B. subtilis*), which has been grown in the incubator for three or four days. The threads after removal are suspended in an empty sterile tube and dried at incubator temperature. They are next suspended in a 1:1000 solution of

mercuric chloride. After five minutes one of the threads is withdrawn with a sterile (flamed) forceps, and divided into equal lengths by means of a sterilized scissors. One of the latter bits is at once transferred to a fresh tube of sterile nutrient substance. The second is gently washed in sterile water to free it from the excess of the sublimate solution; then washed for half a minute in a weak (two per cent.) solution of ammonium sulphide in order to remove any possible coat of mercurial albuminate from about the microorganisms and their spores. This is then rinsed off by second immersion of the thread in sterile water, after which the thread is transferred to a tube of sterile nutrient medium. The same procedures are performed with the remaining bits of thread at the close of ten minutes, twenty minutes, and thirty minutes. Each tube, properly marked, is then placed in the incubator and observations made at the close of the first, second, and third days.

Exercise 19.—Practise one of the methods of surgical disinfection of the hands, inoculating sterile bouillon with scrapings from the hands and material beneath the nails before the process as controls, and similarly planting scrapings from the same sources after each stage of the procedure in order to determine the value of the several steps of the process. In this exercise let the class be arranged in groups of five, each individual planting the preliminary control tubes from the hand and nails. In each group let one man carry out the process in full before making the second inoculation. Let the second wash the hands with soap and hot water, using a sterile hand brush for effectiveness; when, having rinsed the hands in sterile water, let him make inoculations from the epiderm and nails into sterile bouillon. The third should soak his hands for five minutes in a 1:1000 solution of bichloride of mercury, working the disinfectant well into the skin by means of a sterilized hand brush; after which, having rinsed his hands free from the disinfectant in sterile water, let him make similar inoculations from the epiderm and nail scrapings into sterile bouillon. A fourth should soak his hands in a saturated solution of potassium permanganate, rinse off the excess in sterile water, and make similar inoculations. The fifth should soak his hands in a solution (saturated) of oxalic acid, rinse the hands in sterile water, and likewise inoculate tubes of sterile bouillon with scrapings from the skin surface and from the nails. Further, the third man of each group, who has sterilized his hands with the sublimate solution alone, should now soak his hands in a two per cent. solution of ammonium sulphide, brushing it well in, and again rinse off the chemical and make inoculations from the same sources as before. Moreover, the first man, who has carried out the entire process of hand sterilization, should make

deep scrapings from the epiderm, inoculating them as before, in order to determine the degree of penetration of the disinfectant solutions below the surface of the skin. All tubes, properly marked, are now placed into the incubator; and at the usual intervals of one, two, and three days observations made in comparison with the control experiments and the results noted.

Disinfectant Gases.—Disinfection of large spaces, as of sick-rooms, hospital wards, ship-holds, and similar inclosures, is commonly attempted by means of disinfectant gases; the process being completed by washing the floors, walls, and ceilings as far as practicable, and the furniture, when possible, with disinfectant solutions, as of corrosive sublimate or carbolic acid, all comparatively worthless material burned, and all material which can be safely and conveniently subjected to heat thoroughly baked, boiled, or steamed in the usual manner. The gases commonly used for such purpose are formaldehyde and sulphurous oxide. When the method is attempted it is essential for success that the exposure shall be a prolonged one, the room being kept filled with the gas for twenty-four hours, and all cracks and crevices about doors and windows carefully closed with strips of paper pasted over them, or by cotton or rag caulking, to prevent the escape of the gas. Of course, all large openings, as of flues, registers, ventilating openings, as well as doors and windows, are to be thoroughly sealed. All clothes, curtains, carpets, and similar substances which it is desired to have disinfected by the gas are to be hung about the apartment in the thinnest possible layers so as to facilitate thorough penetration. When sulphur is to be used, it is best that water should first be evaporated in the room so as to make the atmosphere as moist as possible, such moisture aiding in the penetration of the gas into any fabric exposed; it is not essential, however, in case formaldehyde is employed, but is not objectionable. In practice it is customary to burn twenty grams of sulphur for each cubic meter of space, the sulphur being either in the form of the sulphur disinfecting candles or in the powder, when it is well to first moisten it with alcohol. Care should be taken to protect the floor and the room contents from the sulphur flame, either by burning the sulphur in candle form floating upon a wide dish of water (the sulphur upon a board or in a shallow dish), or burning the powdered sulphur in a dish set in a large sand or earth bath.

In using formaldehyde, from ten to fifteen cubic centimeters of the commercial solution should be evaporated for each cubic meter of space. A number of forms of apparatus have been devised for the purpose of generating this gas; of these, probably the most efficient are those in which the commercial formaldehyde solution, mixed with glycerine or calcium chloride to prevent its conversion into its solid isomer *paraform*, is vaporized in a metal retort or tube, by means of an alcohol flame beneath, the gas being discharged from the mouth of the apparatus through a tube, by which it may be conveyed through a keyhole or other small aperture into the interior of the room to be disinfected. Or the gas may be obtained by heating the *paraform*, which may be obtained in pastile form in commerce; or it may be produced by the action of a heated plate of copper upon the fumes of wood alcohol. Special types of generators based upon each of the latter methods may be had and will serve the required purpose.

Exercise 20.—Three slips of sterile filter paper or thread are soaked in an active culture of one of the pus germs, as of the *Micrococcus pyogenes aureus*, for an hour. They are then separately inclosed, each in a definite

but varying number of layers of blanket cloth, and distributed to the top, floor, and middle height of a room of known dimensions, which has previously been prepared as above outlined for the prevention of the escape of the disinfectant gas used. Formaldehyde is generated by any convenient form of generator and passed in the above-mentioned proportion into the interior of the room, which is thereafter kept closely sealed for twenty-four hours, then opened and well aired. As soon as convenient the room is entered and the packages containing the infected slips of paper removed and inoculations made therefrom into sterile bouillon, the tubes marked, placed in the incubator, and observations made after the first, second, and third days and the results recorded.

Exercise 21.—The above experiment is to be repeated, substituting sulphurous oxide for the formaldehyde gas.

Résumé.—From the foregoing exercises it should be possible to come to fair conclusions as to the relative merits of the various methods of sterilization suggested; and it is expected, therefore, that the student will in review calculate the actual number and percentage proportion of successful sterilizations accomplished by the class in the various full applications of heat, and by filtration and chemical disinfection.

LESSON III.

PREPARATION OF TUBES, FLASKS, DISHES, ETC.

In the study of bacteria the first object before the investigator is to obtain, isolated from all other forms of microorganisms, a sufficient growth of the particular type to afford opportunity for observation of its characteristics. Such an isolated growth is spoken of as a *pure culture*; where several varieties are more or less mingled in their development, the term *mixed* or *impure culture* is used. The artificial cultivation of these organisms is accomplished by affording them conditions for growth more or less similar to those required by them in nature (nutrient material, a definite range of temperature, moisture, and proper atmosphere). For the purpose of preserving the nutrient material free from other organisms than those purposely implanted upon it, and to aid in maintaining perfect isolation of the various cultures, there is to be provided no little apparatus, the principal forms of which may here be briefly considered.

1. **Test-tubes.**—A large number of test-tubes, the most commonly employed form of container for culture media, should always be available. The ordinary chemical test-tubes may be used for this purpose, but it is preferable that the tubes should be somewhat heavier and made of a glass which will not be corroded by the process of steriliza-

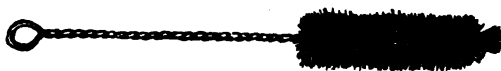


FIG. 11.—TEST-TUBE BRUSH.

tion. They may be plain, or provided with the usual flange about the mouth, as suits the fancy of the individual. A convenient size is five inches in length and five-eighths of an inch diameter; and it will be found advantageous to have a small portion of the outer surface of the upper part of each tube roughened by etching with "white acid," for the purpose of receiving pencil marks in labeling. A larger size is usually provided for the reception of potato cylinders sometimes employed as culture media. Moreover, it is well to have a few very large tubes on hand for use in making anaerobic cultures and as sedimentation tubes in the preparation of solidified blood-serum.

Cleansing New Tubes.—Before the nutrient media are introduced into the tubes the latter are to be carefully cleansed, the mouth of each plugged with a cotton stopper, and the whole sterilized. In preparing tubes which have not been previously used, ordinary washing in water with a test-tube brush (Fig. 11) followed by soaking for from five to ten minutes in a weak solution of one of the mineral acids (as a one per cent. solution of hydrochloric acid) to neutralize any of the remaining alkali employed in manufacture, and by rinsing in clean water, will be found sufficient. The tubes are then to be inverted on the draining board to dry.

Cleansing Old Tubes.—When tubes have been previously used for culture purposes

and perhaps are contaminated by infectious material, the process of cleansing requires more care and detail. It is not free from possible danger, and the student should exercise all the caution and fulfil all the steps indicated in the following directions for its performance. The contaminated tubes having been collected, the operator should remove the old cotton stoppers, grasping them with a strong pair of dissecting forceps (subse-

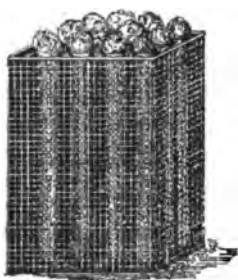


FIG. 12.—WIRE BASKET FOR TEST-TUBES.

quently to be sterilized by flaming), and deposit them at once in one of the laboratory waste jars containing a large amount of one of the disinfectant solutions. This material should be burned subsequently. Each tube, after the removal of the stopper, is to be filled even with its lip with a strong disinfectant solution (as water containing five per cent. each of carbolic and hydrochloric acids), and allowed to stand for from twelve to twenty-four hours, in which interval it is probable the destruction of any living infection will have been accomplished. The contents of the tubes are now poured with as little spattering as possible into one of the waste jars and subsequently burned. As emptied, the tubes are placed in a suitable vessel containing water to which has been added sufficient washing soda to render it distinctly alkaline (two or three percent.), in which

they are to be boiled for an hour. This done, there is no longer danger of infection, and each tube is now thoroughly washed and brushed in clean hot water, the outside surface being wiped clean with a wash-cloth. The tubes are next placed for a few minutes in a weak solution of one of the mineral acids, as in case of new tubes, to neutralize any remaining alkali, rinsed in clean water and placed in inverted position on the draining board to dry.



FIG. 13.—ROUX'S POTATO TUBE.

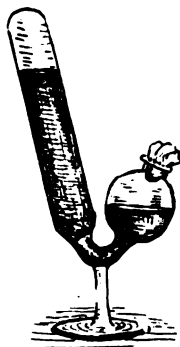


FIG. 14.—FERMENTATION TUBE.



FIG. 15.—FERMENTATION TUBE.

Cotton Plugs.—The next step of the preparation is the application, in the open end of each tube, of a cotton stopper to prevent the entrance of contaminating organisms from without (*v. Ex. 13*). These stoppers should be of such a size that when fitted into the mouth of the tube the latter may be sustained easily when lifted by the hand grasp-

ing the protruding end of the stopper; however, the cotton should not be too closely packed into the tube, and should have been rolled into an even cylinder rather than loosely and unevenly crowded into the tube. Ordinarily common cotton batting is used for the purpose; the only objection, however, to absorbent cotton is its cost. Batting will assume, with proper sterilizing heat to which the tubes are subsequently exposed, a light brown hue which may be taken as a rough indicator of proper sterilization;

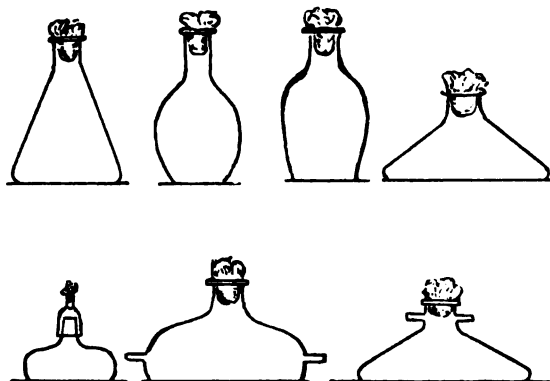


FIG. 16.—TYPES OF CULTURE FLASKS.

however, if higher temperatures should accidentally be attained, batting will present a disadvantage in that a dark oil is driven from the cotton, which will enter the tubes as a vapor and condense upon the inner surface. If permitted to remain it is apt to interfere with the development of inoculations, is unsightly, and should be removed by thorough washing.

Sterilization of Tubes.—After the tubes have been cleaned and the cotton stoppers

adjusted, they are placed in a basket made of wire netting (Fig. 12), and sterilized by baking in the dry-air oven for an hour at a temperature of 140° to 150° C. (v. Ex. 7 and 8). It is not essential that the complete fractional heating should be carried out in this preliminary process (except in special cases, as when the tubes are intended for blood-serum), since after the introduction of the media the thorough sterilization of the latter as well as of the tube will be necessary.

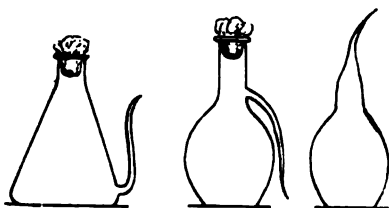


FIG. 17.—TYPES OF DISTRIBUTION FLASKS.

Special Forms.—In preparing tubes for potatoes, if the tubes be of the common form, it is well, before adjustment of the cotton stoppers, to place in the interior of each a small bit of glass tube or rod or a wad of cotton to serve as a support for the potato cylinder in order to prevent the latter from descending to the bottom of the tube, where in the process of sterilization there will collect more or less liquid. This liquid is retained in the tube to afford moisture to colonies of bacteria subsequently grown upon the surface of the potato. A special form of tube is sometimes used to obviate the need

for such rest, as shown in Fig. 13; however, there is added difficulty in cleaning this tube, and it is therefore of questionable advantage.

In the study of gas formation by bacteria, *fermentation tubes* of the same form as those used in urinalysis for sugar fermentation and for the estimation of urea (Doremus' ureometer) are usually used (Fig. 14). The collection arm of such tubes need not be graduated, but the total capacity of the arm should be ascertained so that the amount of gas may be approximately calculated in special cases. An efficient substitute for such fermentation tubes may be extemporized by inverting a small test-tube filled with the medium in a larger one, after the principle of a gas jar in a water-bath (Fig. 15).

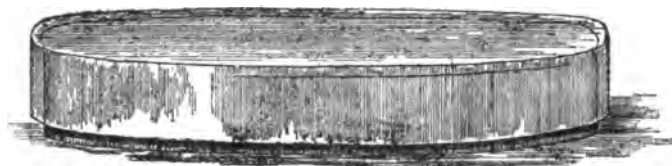


FIG. 18.—PETRI DISH.

2. Flasks.—It is customary to employ flasks as containers for the nutrient media in bulk, distribution to culture tubes being made from time to time as necessity demands. It is best for this purpose to use the small sizes (as those of 250 to 500 cubic centimeters capacity), since, should accidental contamination occur, smaller quantities of the media are endangered. When large amounts of a culture of some special organism are desired, flasks are often used instead of tubes or dishes, and a number of special forms of culture flasks may be obtained from the makers (Fig. 16). So, too, a number of special forms of flasks have been devised for the storage of media and their ready and

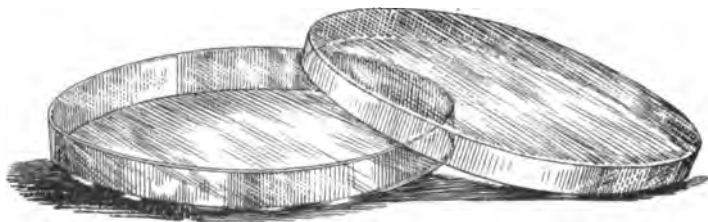


FIG. 19.—PETRI DISH.

safe distribution to tubes (Fig. 17). For general use, however, the ordinary flat-bottomed Erlenmeyer flask is deservedly the most popular, its shape being favorable in cleaning and its broad, flat bottom giving it stability.

Flasks are to be cleaned, stoppered, and sterilized before use in precisely the same manner as detailed for the culture tubes. Sand or shot agitated in the water will facilitate the cleansing of the interior, but it is best to use a long-handled brush obtainable for the purpose.

3. Dishes.—Two types of dishes are in general use—the large form used for potato and plate cultures, and the small Petri dishes.

(a) *Potato Dishes*.—These are large flat dishes, usually about twenty centimeters in diameter and about eight centimeters in height, with straight sides, and fitted with covers of the same shape as the dish itself, but of slightly greater diameter, so as to permit the cover to easily fit down over the dish (Fig. 18). They are used to protect the large slices of potatoes often used as culture material or the sets of plates in plate cultures. Be-

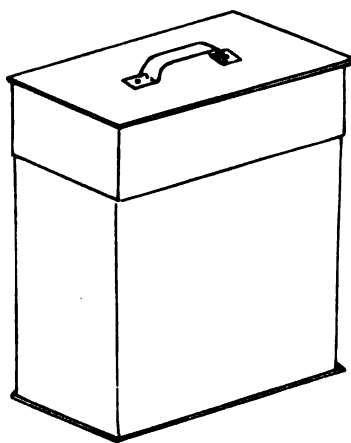


FIG. 20.—METAL BOX FOR HOLDING GLASS PLATES DURING STERILIZATION IN OVEN.

cause of their size and the quality and texture of glass used in their manufacture they cannot well be subjected to heat in sterilization. Therefore, after having been well washed and cleansed after the method detailed for test-tubes (omitting the boiling), it is customary to place in the bottom of each dish a layer of ordinary filter paper (intended to retain moisture for cultures), after which the dish, and, too, the cover, are filled level full with a disinfectant solution (1:1000 solution of mercuric chloride), which is allowed to remain therein for at least one hour (in practice often over night). The sublimate solution is then poured out and the cover at once adjusted, after which the potatoes or plates may be introduced. There are numerous opportunities, in the adjustment of the cover and at time of introduction of potatoes and plates, for the contamination of the interior by organisms from the air, and the protection of the media by such dishes is therefore not to

be compared with that obtained in tubes or flasks; their use, therefore, is by no means so frequent as formerly. Care should be exercised, in the selection of the dishes and covers, that the contact between the cover and the edge of the dish be as perfect as possible.

(b) *Petri Dishes*.—These dishes (Fig. 19), of the same general appearance as the large culture dishes, but much flatter, are usually eight or ten centimeters in diameter and one and a half to two centimeters in height. They were introduced to take the place of the plates employed in the separation of bacteria in impure cultures, and are of particular service, as they permit the examination of the gross colonies of organisms developing in the interior by means of the microscope, the unopened dish being placed upon the stage of the instrument and examined with the low powers of the microscope. The same precautions in the cleansing of these dishes are to be observed as detailed for tubes.

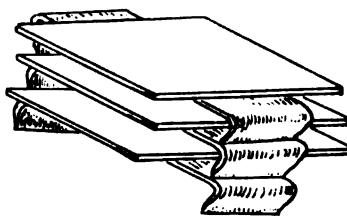


FIG. 21.—SET OF CULTURE PLATES AND PLATFORMS.

The covers are then applied to the dishes and the whole sterilized in the dry-air oven by the fractional method (the whole process should be performed, as there is usually no opportunity for further sterilization after the introduction of the culture medium). It will be found of advantage to fold the dish and cover in wrapping paper before sterilization, the paper being allowed

to remain until they are to be used, in order to prevent accidental separation of the covers from the dishes. Contamination of the interior by bacteria from the surrounding atmosphere is, as in case of the potato dishes (but to a less degree because of the smaller size of the Petri dishes and the better adaptation of their covers), more likely to take place than in the use of culture tubes; but this can in some measure be obviated by the application of a strip of paper or a rubber band, such as is used about bank books, about the outside surface along the line of overlapping of the cover.

4. **Plates.**—Plates of glass, usually four by five inches in size, were formerly much used in the separation of the bacteria of impure cultures to obtain pure cultures, the inoculated medium being spread over the surface of such a plate so that the organisms present might in development grow into well-separated colonies and thus favor the removal of such as may be desired, by means of the needle, to fresh culture tubes. They are occasionally used at present for this purpose, but have been largely superseded by Petri dishes and by the so-called Esmarch tubes. In preparation for use, the plates are cleaned in the same manner as indicated for tubes and are then dried and placed in a metal box with close-fitting lid, in which they are baked in the dry-air oven in the usual manner of fractional sterilization. The box is opened only when the plates are required to be withdrawn for use (Fig. 20). In arranging plate cultures it is customary to place these plates in the large culture dishes above mentioned, usually three in each dish, each supported upon a glass platform and built into a "set," one over the other (Fig. 21). The glass platforms are to be prepared for use in the same way as the plates, but when sterilized should be wrapped separately in paper, which is kept about them for protection until they are to be arranged in the culture.

In addition to the above apparatus there will be required a number of other forms of glassware and other appliances for the preparation and distribution of the culture media and for the prosecution of culture experiments, which may be described to best advantage in connection with the processes in which they are employed.

Exercise 22.—Let the student at this time prepare, according to the preceding instruction, half a gross of ordinary culture tubes, one dozen potato tubes, ten Petri dishes, and clean all tubes, etc., which have been used in previous exercises.

LESSON IV.

CULTURE MEDIA.

A variety of nutrient substances are in more or less general use in the culture of bacteria, and such variety is essential, not merely for the ordinary requirement of growing the organisms, but also for the further purpose of observation of the varying characteristics presented by this or that bacterium upon the different types of media and furnishing data useful for its identification. Of the different media suggested, the most important for routine use are *potatoes*, *bouillon*, *gelatinized bouillon* ("gelatine"), *agarized bouillon* ("agar"), *peptone solution*, *glucose-*, *lactose-*, and *saccharose-bouillon*, *blood-serum*, *milk*, and *litmus-milk*. In the preparation of these media it is important, inasmuch as from variation of their constitution there may follow important alterations of the characteristics of the bacteria grown upon them, that uniformity of manufacture should be sought in order that the records of different individuals engaged upon the same microorganisms may be uniform and comparable. It is desirable, too, that all results observed as to the growth of any bacterium upon the standard or upon special modifications of the common media should be recorded with clear indication of the exact composition of the nutrient medium employed, as well as the other conditions of growth prevailing. The reaction of the medium is an especially important feature; in which matter it is to be recommended that the standard reaction adopted by the Bacteriologic Committee of the American Public Health Association be followed. It is to be understood, except as may be indicated in the following instructions, that the reaction of all media described should be adjusted to this standard: that to phenolphthalein (a more delicate reaction indicator than litmus) the reaction of the medium will be +1.5 (acid 1.5 per cent.—that is to say, each one hundred cubic centimeters of the medium contains a sufficient excess of acid elements to require for their neutralization 1.5 cubic centimeters of a normal sodium hydroxide solution). Although this is accepted as a standard reaction, it will not infrequently be observed that variations presenting greater or less percentage of acid, or which are actually alkaline, will be more favorable for the growth of individual forms of bacteria; and a certain number of such modified media may be advantageously kept in stock for special work. The reaction is to be indicated by the percentage quantity of acid or alkali (hydrochloric acid or sodium hydrate) in normal solution required for the neutralization of one hundred cubic centimeters of the medium, the mark + indicating acid, the mark —, alkaline reaction. Thus, bouillon, sufficiently acid to require for the neutralization of one hundred cubic centimeters 0.5 cubic centimeter of a normal solution of sodium hydroxide, would be indicated as +0.5; a bouillon of alkaline reaction sufficient to require for the neutralization of one hundred cubic centimeters 0.75 cubic centimeter of a normal solution of muriatic acid would be represented as —0.75 reaction. For the determination and adjustment of reaction there will be required the following solutions: (a) a normal solution of sodium hydroxide in distilled water (40 grams NaOH, 1000 cubic centimeters water); (b) a decinormal solution of sodium hydroxide (4 grams to 1000 cubic centimeters

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distilled water); (c) a decinormal solution of hydrochloric acid (3.65 grams to 1000 cubic centimeters distilled water); (d) a normal solution of hydrochloric acid (36.5 grams to 1000 cubic centimeters distilled water); (e) a 0.5 per cent. solution of phenolphthalein in fifty per cent. alcohol. In the reaction-correction of a given medium (supposing it to be excessively acid), the volume is first to be corrected to a definite amount. This is most readily done by correction of weight, the weight of a given volume of the medium having been previously determined by practice or calculation for the temperature of the room (20° C.). If the weight be less than it should be, water is to be added to the required amount; if excessive, the excess is to be evaporated by boiling. This attended to, five cubic centimeters (or the equivalent weight, preferably) are withdrawn, forty-five cubic centimeters of distilled water added, and this boiled for several minutes in a porcelain evaporating dish to expel any carbon dioxide present. One cubic centimeter of the phenolphthalein solution is now added, and the mixture titrated while hot with the decinormal sodium hydroxide solution from a burette provided with a glass stop-cock. The alkaline solution is added slowly, drop by drop, with constant stirring, until a distinct pink color results. The number of cubic centimeters used of the decinormal sodium hydroxide solution is now read from the burette markings, indicating the quantity of solution of this strength necessary for the complete neutralization of five cubic centimeters of the medium. If there were originally 1000 cubic centimeters of the medium, 995 cubic centimeters would remain; and for the complete neutralization of this amount the quantity of decinormal sodium solution can readily be calculated. Addition of so large an amount of fluid as this would entail would, however, be complicating in its effect; and to avoid this, one-tenth the same quantity of a normal solution is substituted. However, the adopted standard is not the absolute neutral point, but a reaction of such acidity as to require for the complete neutralization 1.5 cubic centimeters of normal sodium hydroxide solution for each one hundred cubic centimeters of the remaining medium. Therefore, from the total quantity of the normal alkaline solution calculated to be required for the complete neutralization of the remaining 995 cubic centimeters of the medium are to be deducted 14.9 (9.95 by 1.5) cubic centimeters; the remaining number of cubic centimeters of the normal alkaline solution are then to be added and diffused to render the medium of the standard (+1.5) reaction. If the medium was originally alkaline, on the addition of the phenolphthalein it should have been titrated in the same way with a decinormal solution of hydrochloric acid until the discharge of its pink color. The number of cubic centimeters thus used is employed as basis for calculation of the quantity of the same strength acid solution necessary for the neutralization of the remaining medium. One-tenth this amount, plus 1.5 cubic centimeters additional for each one hundred cubic centimeters of the remaining medium, is added in the form of the normal solution of the acid in order to produce the standard reaction.

I. CARBOHYDRATE MEDIA.

1. *Potatoes*.—These vegetables are most frequently employed as a culture medium for the chromogenic bacteria and for a few other organisms, as those of typhoid fever and of glanders, which produce rather characteristic growths upon them. Their preparation is simple, consisting merely of proper cleansing, cooking, and sterilization, inoculations being made upon the cut surface of the vegetable. It is not customary to modify the natural (acid) reaction of the potato in such use. They may be employed either in dish or tube cultures, preferably the latter.

(a) Dish Cultures.—

Exercise 23.—Select potatoes of as regular shape as possible, without blemish and with as few eyes as possible, each student preparing two potatoes. Wash them in running water, using a stiff hand brush for effectiveness. The eyes and any suspicious spots are next cut out freely, after which the potatoes are immersed in a dish containing 1:1000 solution of bichloride of mercury for an hour. They are then put in the steam sterilizer in a tin bucket with perforated bottom ("potato bucket") and cooked for three-quarters of an hour or an hour at 100° C. They are not to be removed from the sterilizer, but are again steamed at the same temperature for fifteen minutes upon the second and third days. A potato dish should in the mean while be prepared for their reception

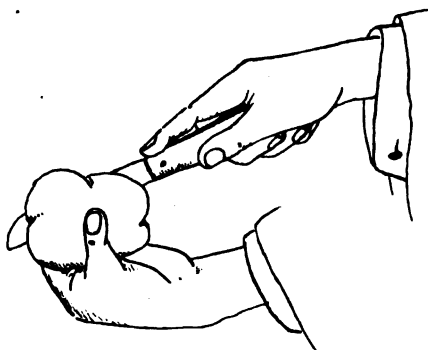


FIG. 22.—SECTION OF POTATO INTENDED FOR DISH CULTURE.

at the close of the process of sterilization upon the third day (*v. instructions, Ex. 22*). The hands of the operator are now to be thoroughly washed with hot water, soap, and brush, and soaked for five minutes in a 1:1000 solution of mercuric chloride and a potato knife, a flat-bladed knife such as is commonly sold for use in the household for paring potatoes (*Fig. 22*), is sterilized by flaming (*v. instructions, Ex. 6*) if it has not previously been prepared by baking or boiling. The opera-

tor, holding the potato with the thumb and fingers of the left hand grasping its shortest diameter, divides it into halves, drawing as little of the blade as possible through it and retaining the blade in position between the cut surfaces without separating the two halves. An assistant raising the cover of the culture dish, the potato is deposited therein in position so that, as the two halves are separated by proper movement of the knife, the cut surfaces will fall apart and remain uppermost. The dish is now temporarily closed, and the second potato is in like manner to the first divided and placed in the dish with its cut surfaces exposed. In these operations no more exposure of the interior of the dish is allowed for entrance of organisms from the air than can be avoided; and obviously the procedure should not be performed where any atmospheric draughts prevail. In order that the student may obtain some idea of the objectionable features of such cultures and the value of the method, this first

dish may profitably be left closed and uninoculated for the purpose of observation. It is very common that in spite of all care exercised the potatoes soon show the growth of numerous colonies, infection having taken place from the air which was inclosed in the dish (unsterilized air) at some time when the cover was removed, or entered the closed dish through unobserved imperfections in the line of application of the cover to the edge of the dish (should slight currents be in some way induced through such small apertures). This last possible method of convection of infection may be prevented by applying a thick layer of vaseline to the margin of the dish before applying the cover.

(b) *Potato Tubes.*—

Exercise 24.—One dozen potato tubes have been previously prepared



FIG. 23.—CULTURE TUBE CONTAINING CYLINDER OF POTATO RESTING ON SMALL GLASS ROD.

and once heated in the oven (Ex. 22). The same care as to the selection of suitable potatoes is to be observed as in the preceding exercise. They are then carefully washed and rinsed in running water. The ends of each potato are cut squarely off, and with a cork-borer of a diameter slightly less than that of the tubes (or with a knife if the cork-borer be not at hand), cylinders of the potato substance are cut from each and placed in water. After half a dozen such cylinders have been obtained, each is to be cut in an oblique fashion so that each resulting piece presents a round, flat end and a large oval beveled surface for exposure in the tube (Fig. 23). These pieces are now left in running water for several hours (or over night if convenient), the washing preventing their discoloration in the subsequent sterilization. Thereafter each bit is introduced into a tube, beveled surface uppermost, the flat end resting on the bit of glass rod (or wad of cotton or other rest) placed in the bottom of the tube, and the

cotton stopper is readjusted. When all the tubes are thus filled, they are placed in the steam-bath (at 100° C. for thirty minutes, repeated for fifteen minutes on the second and third days) or in the autoclave (120° C. for thirty to forty minutes) for sterilization.

(c) Occasionally, after the potatoes are washed and pared they are cut into broad, thin slices, which are placed in Petri dishes and sterilized there just as if in tubes. In this way extensive surface exposure may be obtained, of material advantage in the separation of mixed cultures.

Further, potatoes, after being cleansed and boiled, may be mashed and the result-

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ant paste may be spread in dishes or introduced into tubes if desired. In this method of preparation one may take advantage of the opportunity afforded for adjusting the reaction of the medium, by boiling the paste in a sodium hydrate solution to correct the reaction uniformly through the mass.

Glycerine potato tubes differ from the above ordinary tubes merely in that a five per cent. solution of glycerine is introduced into the tube before sterilization, up to the level of the lower surface of the potato; from which a small amount of the glycerine diffuses gradually through the potato and over its surface. Tubercle bacilli may be grown upon such a preparation.

(d) *Elsner's Medium*.—This is a modification of Holz's *potato gelatine* (used for the same purpose as Elsner's medium), designed as an advantageous medium for the isolation of the *Bacillus typhosus* and *Bacillus coli communis* from the variety of organisms with which they are apt to be associated in the dejecta, and from each other. Upon it the common saprophytic germs are almost completely inhibited, and the typhoid fever organism develops at a later period than *Bacillus coli* and with such differences of appearance in the colonies as to make possible the distinction of one from the other, thus facilitating their separation. The medium may be made as follows: Five hundred grams of pared potato are grated finely and the pulp placed in the refrigerator, in a porcelain dish, over night. The following morning the juice is expressed from the pulp and filtered several times through a layer of absorbent cotton or through animal charcoal (preferably the latter). The filtrate should now be titrated with decinormal sodium hydroxide solution to determine its reaction and the amount of water which will be required to be added to reduce its acidity to the standard. In this quantity of water is now boiled the amount of gelatine required to make a ten per cent. proportion of the gelatine when the potato juice shall have been added, the reaction of the gelatine being corrected to neutral point after it has been dissolved (and before adding the potato juice) by means of normal sodium hydroxide solution. This done, and the bulk of the fluid corrected for evaporation to that necessary to properly dilute the potato juice, the latter is slowly added to the gelatine and mixed, and the whole boiled for five to ten minutes, filtered, and distributed to tubes or flasks, and sterilized in the usual interrupted manner in steam. Thus far the medium constitutes *Holz's potato gelatine*. *Elsner's medium* is made from this by adding freshly when required for use one cubic centimeter of a sterilized solution (ten per cent. strength) of potassium iodide to each ten cubic centimeters of the medium. It will be found advantageous for incubation at body heat that one-half the required amount of gelatine be substituted by a corresponding amount of agar (one per cent. of agar).

2. A number of other carbohydrate media are employed for culture purposes, as carrots, turnips, apples, bread-paste, etc. They are used for the most part for the cultivation of the moulds and of chromogenic forms of bacteria, but are scarcely of sufficient importance to be further detailed here. The various sugars as culture media will be noted in connection with the media in which they are usually used.

II. PROTEID MEDIA.

By experience it has been found that nutrient media containing proteid substance are upon the whole the most favorable for the culture of the greatest number of known bacteria, particularly the pathogenic forms; and it is probable that with further study the selection of such material will be far more exact than at present apprehended. The usual source at present is adult beef; yet it is probable, from the comparatively limited

experience of bacteriologists in this direction, that much can be done in selective cultivation by substituting the proteids of other animals, even from man. The development of this field presents possibilities for investigation. The albumins at present utilized are for the most part of the nature of albumoses, peptones, and serum albumin; and their preparation is largely empiric, and the precise composition of the products a matter of uncertainty. The amount of albuminous substance in ordinary bouillon and its derivatives is in reality much less than was originally contemplated in its manufacture; and it is probable that the many complex organic and inorganic salines and derivatives preserved in its manufacture constitute the most important feature of the substance. One sees but little difference practically, for example, in the cultural qualities of the media made from the commercial beef extracts and those made from the fresh beef; yet it is well known that these extracts contain comparatively little of the meat albumins and are largely composed of the various salines and extractives. In blood-serum, of course, the albumin of the serum is preserved; and this substance more nearly than the bouillon preparations represents a natural proteid medium. In bouillon preparations the loss of the albumins is sought to be corrected by the addition of commercial peptones (really albumose for the most part); and solutions of the latter will be found to possess every practical value of the more complexly arranged bouillon.

1. **Bouillon.**—Bouillon is the nutrient medium commonly selected when large, massive cultures of bacteria are required to be grown, or when it is desired to have the bacteria in liquid surroundings, to facilitate observations as to their power of movement, their intimate relations, or their chemical activities; or when they are intended for animal inoculation. It is prepared either from the fresh meat or from the commercial beef extracts. It is very much more convenient to make the preparation from the latter, and so far as the value of the product as a nutrient medium is concerned, it is apparently in no way less advantageous than that made from the fresh meat. It possesses one important advantage, moreover, in that it is less likely to contain the meat sugars uniformly met in the meat bouillon, rendering it preferable in exercises intended to demonstrate reaction changes and gas formation by bacteria; and in its manufacture it is more easy to maintain a definite and uniform composition.

(a) When making bouillon from the fresh beef, the meat is obtained from the butcher as fresh as possible; it should be free from fat and should be finely chopped or ground. The meat pulp thus procured is soaked over night in water (preferably boiled water—in proportion of 500 grams of the pulp to 1000 cubic centimeters of water), being kept in the refrigerator in order to prevent the development of the bacteria present in the meat. The following morning the red meat infusion is strained off through a piece of cheese-cloth, the pulp being compressed if necessary to obtain a full liter of the liquid. If less than a liter be obtainable, it is customary to add water to make up the amount to this quantity. The infusion is next heated moderately (between 50° and 60° C.) in a suitable vessel (as a granite-ware stew-pan), and ten grams of dried peptone and five grams of sodium chloride are added, and with constant stirring with a clean glass rod, thoroughly mixed and dissolved in the warm infusion. When the peptone and salt are dissolved, the preparation is boiled sharply, with constant stirring, until all the albumins coagulable by heat are separated and the liquid is of a clear, amber color. It is then filtered through paper; and should the filtrate be at all turbid, it should be reboiled and again filtered. The filtrate is now cooled to room temperature and the amount corrected to one liter, after which it is titered with decinormal sodium hydroxide solution and the reaction adjusted. It is again

to be boiled and filtered, after which it may be distributed to tubes or flasks and sterilized by the fractional method in steam (100° C.), or in the autoclave (120° C.).

(b) When the bouillon is to be made from beef extract, 1000 cubic centimeters of water are placed in a clean stew-pan, and five grams (various proportions have been recommended, from two to five grams) of Liebig's, or other standard brand of extract, added, together with ten grams of dried peptone and five grams of sodium chloride. Gentle heat is applied to facilitate solution, the preparation being constantly stirred with a clean glass rod. When solution has been effected, the liquid is well boiled for from five to ten minutes, with constant stirring. It is then filtered through paper, cooled, and the volume corrected to one liter, after which the reaction is determined and adjusted in the usual manner. It is thereafter again boiled and filtered, and distributed, when it is sterilized in the usual manner.

Exercise 25.—Each student prepare one liter of bouillon from beef extract as above directed, dividing the product as follows: 500 cubic centimeters for use in the manufacture of gelatine and agar; 300 cubic centimeters for manufacture of glucose-, lactose-, and saccharose-bouillon; and 200 cubic centimeters for use as plain bouillon, distributing of the last, sixty cubic centimeters to a dozen tubes, and placing the remainder in a stock-flask for future distribution as needed. Measure out 1000 cubic centimeters of distilled water into a clean granite-ware stew-pan. Add five grams of Liebig's extract, which has been weighed out on a balanced slip of paper (paper and contents thrown into the water), ten grams of dried peptone (Witté's), and five grams of table salt. Warm and stir until the above ingredients are dissolved. Boil for five to ten minutes to separate any coagulable albumins. Cool to room temperature and correct volume to original amount by addition of boiled distilled water. Determine reaction and adjust to standard. Heat by strong boiling or in autoclave for ten or fifteen minutes, and allow to cool for separation of excess of phosphates. Filter and distribute, and sterilize by fractional method in steam.

(c) *Glucose-, Lactose-, and Saccharose-bouillon.*—These preparations are used mainly for the determination of fermentative qualities of various organisms, the destruction of the carbohydrates setting free carbon dioxide. For their manufacture a bouillon known to be free from meat sugar should be prepared, to which is added one per cent. of glucose for glucose-bouillon; one per cent. of lactose for lactose-bouillon; or the same proportion of saccharose for saccharose-bouillon. The presence of meat sugar in a bouillon may be determined by testing with Fehling's solution or by inoculating a fermentation tube filled with the sample to be tested, with a gas-forming organism, like the *Bacillus coli*, incubating it at body temperature for twenty-four hours, when gas formation will be found to have taken place if sugar be present in the bouillon. Usually, bouillon made from beef extract is free from this substance and is therefore of advantage in the preparation of these media. If one will inoculate a flask of ordinary bouillon with the *Bacillus coli communis* and allow it to grow for several days at incubator temperature, the sugar will be destroyed thereby; and by subsequent steriliza-

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tion and filtration the bouillon will be rendered suitable for the preparation of these special media.

Exercise 26.—Of the bouillon made by the student in the preceding exercise, after testing with Fehling's solution to determine that no sugar is present, withdraw three portions of one hundred cubic centimeters each, the amount retained for the preparation of the sugar bouillons. (If these amounts be withdrawn from a general stock, the remaining portion is to be resterilized.) Each of these portions is to be placed in a clean beaker or flask, and one gram of glucose added to the first and a like amount respectively of lactose and saccharose to the second and third. Aided by gentle warming and stirring with a clean glass rod, these substances are dissolved, and the product placed in fermentation tubes or retained in the flasks, and sterilized.

DISTRIBUTION OF LIQUID MEDIA TO TUBES, ETC.

The distribution of fresh blood-serum, bouillon, peptone solution, and other nutrients liquid at ordinary temperatures, and of gelatine and agar preparations after liquefaction by heat, is performed in the same way whether the destination of the material be to tubes, flasks, or dishes. In case of blood-serum intended for the preparation of a clear jelly, the utmost care must be taken to avoid contamination by microorganisms, since in the further steps of its preparation it will not be possible to depend certainly upon the low degree of heat alone permissible for sterilization. In case of gelatine, too, inasmuch as this substance, if overheated or heated too frequently or too long, is liable to alterations and loss of its power of setting, caution should be exercised to prevent infection of the finished preparation. With the other forms of nutrient media, including blood-serum intended for the opaque white solid serum, there need not be particular care in the process. When small quantities are to be distributed, probably the most convenient method will be found in the withdrawal of the liquid or liquefied medium from the stock-flask by means of a pipette of fifty to one hundred cubic centimeters capacity (if convenient the pipette should be sterile, but this is not essential). It is convenient here that an assistant should hold the tubes, withdraw the cotton stoppers for introduction of the medium, and thereafter replace the same in the tubes. However, a stand can easily be made by boring holes of suitable diameter and depth into a block of wood, to serve as a stand for the tubes in the process, which can then be readily conducted by one person. In introducing the medium into the tubes it should not be permitted to come in contact with the upper part of the inner surface, lest after the application of the cotton stopper the latter may be contaminated and perhaps glued to the glass.

A convenient method is to distribute from a separation funnel (or an ordinary funnel covered by a plate to prevent contamination, as shown in figure 24), a rubber tube fitted with a pinch-cock connecting a glass pipette to the lower end of the funnel.

If care is taken there is no objection to pouring the medium from the flask into the tubes, first having flamed the lip of the flask to destroy bacteria which are apt to be lodged upon it.

Where great caution is to be observed, as in the distribution of fresh blood-serum, siphonage is to be recommended, the siphon arranged as shown in the accompanying

diagram (Fig. 25). The principle of the ordinary chemical wash-bottle, too, may be easily utilized for distribution from stock-flasks, the tube carrying the compressed air into the flask being provided with a cotton plug to prevent the entrance of contaminating organisms, and force being applied from any convenient source, as a reversed siphon, compressed air tank, carbonic oxide tank, or from the mouth (Fig. 26). Of course, in all cases in which it is essential to prevent contamination the tube of the siphon or other appliance introduced into the medium should have been properly sterilized.

The distribution flasks devised by various workers, several of which are shown in figure 17, are convenient, but by no means essential.

In tubing it is customary to put about five cubic centimeters of medium into

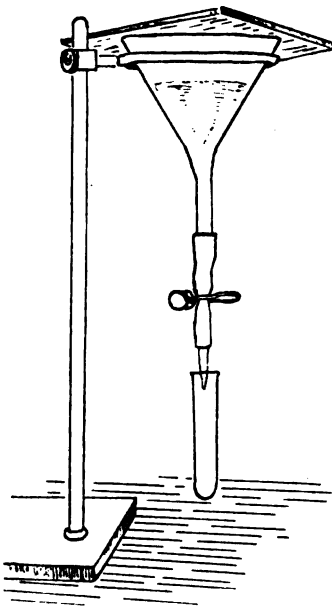


FIG. 24.—DISTRIBUTION OF LIQUID MEDIUM FROM COVERED FUNNEL TO CULTURE TUBE.

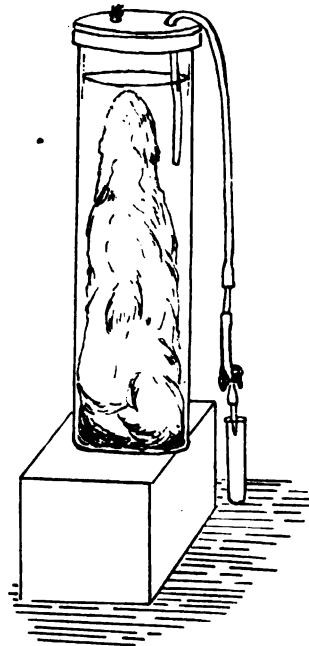


FIG. 25.

each tube, approximation of this amount being sufficient for ordinary work. It is well, however, to provide a number of tubes containing exact amounts, for special purposes. For dilution experiments four or nine cubic centimeters will be found convenient quantities. Tubes of the same size having been selected, the desired number of centimeters of water are placed in one tube, and from the level of this the other tubes are marked and the medium subsequently filled in to the mark upon each.

2. Peptone Gelatine ("Gelatine").—This medium consists of the ordinary bouillon to which has been added from ten to fifteen per cent. of the best sheet gelatine

in order to give it a solid consistence. The especial value of a solid over a liquid medium rests in the fact that it does not conduce to the free diffusion of the bacteria inoculated upon it, but permits their development into definite and more or less isolated colonies, presenting gross characteristics apt to be lost by diffusion through a liquid, thus facilitating the recognition of the organisms and their separation into pure cultures. There is comparatively little nutrient value in the gelatine, but it affords an additional value in being liquefied by the proteolytic power of a large class of bacteria, thus giving rise to a broad classification of these microorganisms into the two great classes of *liquefying* and *non-liquefying bacteria*. The solid consistence of gelatine is lost at about 30° C., and its usefulness is therefore impaired for bacteria requiring incubation at a temperature above this. This disadvantage may be obviated in some measure by combining with it a small amount of agar. In the preparation of gelatine it should be kept in mind that superheating or too prolonged or too frequent heating, even at the ordinary temperature of the steam-bath (100° C.), may convert the gelatine

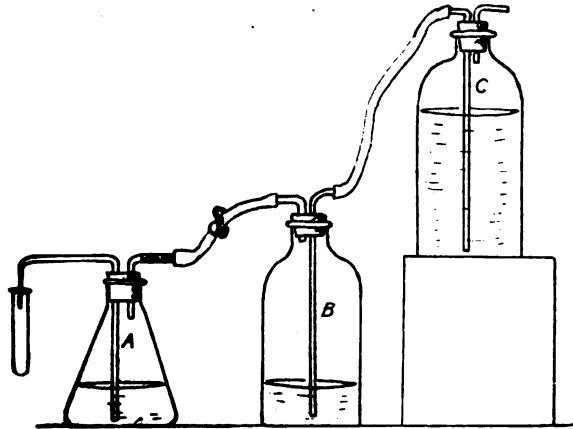


FIG. 26.—DISTRIBUTION TO CULTURE TUBE OF LIQUID MEDIUM FROM FLASK A, BY PRESSURE FROM SIPHON FLASK B.

into paragelatin and destroy its power of congelation; and caution in this connection is essential for the best results. In its manufacture, if it be necessary to freshly prepare the bouillon, the process may be shortened by neutralizing it immediately after solution of the peptone and salt (litmus paper may in this preliminary neutralization be used as a sufficiently exact indicator), and then adding the gelatine. When prepared, tubed, and sterilized, those tubes intended for surface inoculation should be placed in a slanting position, so that the medium may set with a larger surface for exposure. The congelation is best secured by rapid cooling of the medium on a block of ice, or in the refrigerator, or in a bath of ice-water.

Exercise 27.—(Prepare bouillon as just suggested if not on hand.) Two hundred and fifty cubic centimeters of the bouillon prepared in exercise 25 are measured into a clean stew-pan. Add twenty-five grams of the best sheet gelatine, broken or cut into small pieces, and heat gently

to dissolve. When the gelatine has been dissolved, cool the preparation to 60° C. or less, and add thereto one-half the white of an egg dissolved in twenty-five cubic centimeters of boiled water (ten per cent. solution of commercial egg-albumen may be substituted), mixing it well with the liquid by stirring. Boil for ten or fifteen minutes to coagulate the egg-albumen and to reduce the volume by evaporation to the original amount or less. Filter hot through a moistened filter paper which has been folded after the manner customary in the chemical laboratory, to separate the coagulated egg-albumen (albumen added and coagulated in order that solid particles which might interfere with the transparency of the product may be caught in the meshes of the coagulum and thus be removed); correct volume (correct to original weight, *i. e.*, weight of bouillon plus weight of gelatine). While yet hot determine reaction and adjust to standard. If medium becomes clouded, again boil and filter. Distribute half to tubes, and sterilize by fractional steaming; retain remainder in small stock-flask and sterilize in the same manner.

3. Peptone Agar-agar ("Agar").—This medium is made by the addition of two per cent. of agar-agar (a vegetable gelatine derived from an alga found on the Eastern Asiatic coast) to bouillon. The purpose of the addition of this substance is the same as that of the addition of gelatine—*i. e.*, the solidification of the mass; but as the medium after preparation is not liquefied by temperatures below 85° to 90° C. (again setting at about 40° C.), it is better suited than gelatine for culture of organisms requiring incubation. In the manufacture it is necessary that the agar should have been thoroughly dissolved into a limpid liquid for ready filtration; this is accomplished by prolonged boiling (one or two hours), its property of congelation not being interfered with by such exposure to heat. The presence of excess of acid in the material to which the agar is added is more or less harmful to its solidifying power, for which reason the bouillon should have been neutralized (litmus reaction sufficient) before the agar is added. The product is not quite so clear as gelatine at best. This medium is not affected by the proteolytic ferments.

Exercise 28.—Five grams of finely chopped or ground agar are boiled for one or two hours in one hundred cubic centimeters of water in a beaker or other vessel, water being added from time to time to prevent drying. To this is added at the close of boiling 250 cubic centimeters of the bouillon prepared in exercise 25 (or same amount of bouillon freshly prepared up to the stage of sterilization, if stock bouillon be not on hand); and the mixture boiled until evaporation has reduced it approximately to the desired weight (weight of bouillon plus five grams of agar). It is now cooled to 60° C., or less, and one-half the white of an egg dissolved in twenty-five cubic centimeters of boiled water added and stirred into the preparation. Reboil for ten or fifteen minutes to coagulate the egg-albumen and filter through a folded and moistened filter paper. If it is

feared that filtration will be slow, the filtration funnel and receiving flask, a cover being adjusted to the funnel to prevent the water of condensation from dripping into its contents, may be placed in the autoclave, where the temperature is raised to 110° to 115° C. Or the hot-water filtration bucket suggested by Matlock may be employed to keep the preparation well liquefied during the period of filtration (Fig. 27). After filtration the weight should be exactly corrected (weight of bouillon plus five grams of agar) by evaporation of excess by further boiling or by addition of boiled water if deficient; after which the reaction is to be determined and adjusted to standard. Should the medium now become turbid, it is to be reheated and again filtered, after which half

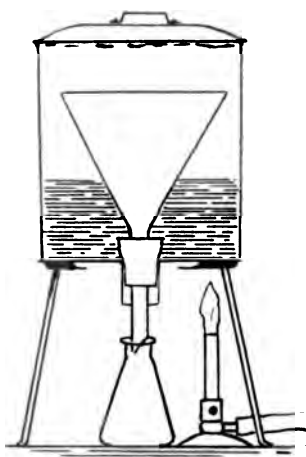


FIG. 27.—HOT-WATER FILTRATION BUCKET.

of it may be transferred to tubes and the remainder retained in the stock-flask, and sterilized either in the autoclave or in the ordinary steam-bath (by fractional steaming in the latter case). Those tubes intended for surface inoculations are to be allowed to solidify in a slanting position, to increase surface exposure, the remainder allowed to cool in erect position, for use in stab cultures and for plate cultures.

Modifications of Agar.—

(a) *Gelatine-agar*.—For plate cultures agar is not as well suited as gelatine, as it does not spread as evenly as the latter unless heated to a temperature fatal to bacteria which it is desired to have diffused through the melted mass; and on the other hand gelatine cannot be used for either tube or plate cultures if it be desired to subject the cultures to the body temperature in the incubator (37.5° C.), because it becomes liquid at 30° C. In order to adjust these difficulties and to provide a medium capable of indicating the liquefying power of bacteria in cultures grown at incubator temperature, a mixture of gelatine and agar has been proposed, which serves the desired end fairly. It may be made by adding the liquefied agar to liquefied gelatine in equal proportion; or in the manufacture there may be added to bouillon but one per cent. of agar, after this has been melted adding five or six per cent. of gelatine. In other respects the preparation follows the steps indicated in the preparation of either agar or gelatine. The medium retains its solid consistence at incubator temperature; and while liquefaction does not take place with the same readiness and to the same degree as in case of the pure gelatine, it occurs sufficiently to be indicative.

(b) *Glycerine Agar*.—This medium is especially valuable as a nutrient in the cultivation of tubercle bacilli and a few other organisms. It is prepared by adding five per cent. of glycerine to ordinary agar after it has been completed, all but the sterilization.

(c) *Lactose-litmus Agar*.—This modification is used in the study of acid production by bacteria and as a means of differentiation between acid-producing bacteria and organisms not possessed of the power of thus converting the sugar. It is prepared by adding to agar of slightly alkaline reaction (-0.5) two per cent. of lactose, and after sterilization tinting the liquid medium before it congeals to a pale blue color with a sterile litmus solution. Acid-producing bacteria growing upon this medium cause a pinkish discoloration of the mass in and about the colonies, contrasting with the blue color of the rest of the medium and the colonies of bacteria not possessing the acid-producing power.

4. *Peptone Solution*.—This is often and with advantage used in place of bouillon. It is made by dissolving ten grams of dried peptone and five grams of table salt in one liter of distilled water; after which it is to be filtered, distributed, and sterilized. It is not essential to adjust the reaction, as it is almost constantly neutral or nearly so. The medium is especially favorable for use in the study of indol and phenol production.

Rosolic Acid-peptone Solution.—This substance serves as a means of determining reaction changes produced by bacteria. It consists of the above peptone solution, to which has been added two per cent. of a 0.5 per cent. solution of rosolic acid in eighty per cent. alcohol. After diffusion of the dye through the peptone solution, the medium is distributed to tubes and sterilized by fractional steaming. It is of a pale pink color, which is intensified by alkaline changes and discharged by acid alterations produced in the culture.

5. *Blood-serum*.—This substance, while an important nutrient for a large number of bacteria, is of especial value for the cultivation of various pathogenic germs which grow but feebly or not at all upon the ordinary media. The serum is generally obtained from the blood of beeves slaughtered in the abattoir, or of the horses used in the places of antitoxin manufacture; occasionally the blood of smaller animals, as calves, sheep, and the laboratory experiment animals, is used. Exceptionally the serum is taken from human blood, as that from the placenta. There are differences in the values of the serum derived from these various sources, but for the ordinary scope of bacteriologic work they are not of importance. As already suggested, however, this feature is one to which future study is likely to add considerable interest.

The serum may be used either in its liquid state or after solidification by heat. After coagulation by heat it cannot again be liquefied, and is therefore a suitable medium for cultivation at incubator temperature. The same fact, however, indicates the impossibility of this substance as a medium for plate cultures, although mixtures of serum with agar or gelatine (from these, however, the coagulable albumins are lost in the manufacture) are sometimes employed for the purpose. Solidified blood-serum is liquefied by the proteolytic action of bacteria, but to a less degree than gelatine.

(a) The collection and preparation of solidified serum, especially by the older method, requires no little care and time; and the busy practitioner may without disadvantage for the cultivation of any of the organisms requiring attention in clinical work, as the diphtheritic germ, substitute for the serum the whole blood. Before it has time to clot the blood is distributed to a number of tubes, coagulated by exposure to a temperature of 90°C . in the oven, and then sterilized in the common fractional manner in the steam-bath. The opaque black surface of the medium thus made contrasts sharply with the pale or white colonies of most of the organisms and is not disadvantageous; nor is the cultural value of the whole blood appreciably less for the majority of bacteria than that of the solidified serum.

(b) When the serum is to be used as a liquid medium (instead of bouillon), it

must be kept in mind that it is impossible to subject it to temperatures capable of certainly sterilizing it, and that therefore the utmost precautions must be observed in its collection and distribution to tubes to prevent contamination. The same care is to be exercised in the collection when the older method of solidification and sterilization is to be pursued, as the clear, straw-colored appearance of the product cannot be preserved if it should become necessary to sterilize the substance at a temperature above 80° to 90° C. If, however, the white opaque solid (like boiled white of egg) is intended, no particular precaution is essential, as this product may be sterilized without injury in steam in the ordinary fractional manner. Even in such case some care that no decomposition should have taken place before preparation of the medium should be had, lest the natural faint alkalinity of the blood be altered and other important constitutional changes have been induced. Usually no attempt at adjustment of the reaction is made, the natural reaction of the serum being sufficiently constant and perhaps in some cases a favorable condition for the growth of bacteria inoculated upon it.

In conditions permitting care in collection these details should be followed in

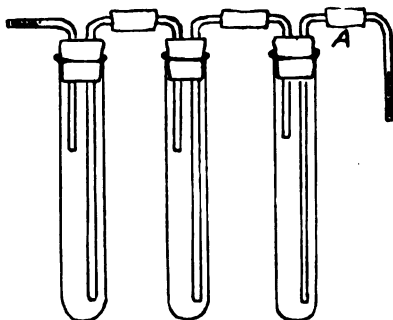


FIG. 28.—SERIES OF TUBES ARRANGED FOR COLLECTION AND SEDIMENTATION OF BLOOD-SERUM.

order to preserve the natural asepsis of the blood: A tall, narrow jar, with cover which may be sealed against the entrance of atmospheric contamination, is sterilized in a large autoclave, or by boiling; or the sterilization may be performed by disinfecting solution (as of mercuric chloride, or of carbolic and hydrochloric acids), with subsequent rinsing with well-boiled water, alcohol, and finally ether. It will be found convenient if the cover of the jar has had two openings drilled into it, these being closed after sterilization by sterile cotton plugs. A suitable rubber tube, five or six feet in length, and of diameter easily entering one of the openings in the cover of the jar is similarly sterilized and rinsed in well-boiled water and wrapped in a sterile towel

or other protective until required for use. In one end of this a glass canula should have been fitted for insertion into one of the jugular veins of the animal from which it is expected to obtain the blood. At the abattoir the free end of the rubber tube is slipped into one of the openings of the cover of the jar and fastened so as not to be easily withdrawn, the end attached to the canula being meanwhile kept under cover. When the animal is felled, it should at once be drawn up by the hind-legs until the head swings almost clear of the floor. The head is then dragged to one side and the exposed side of the neck cut. At once the canula, with as little exposure as possible, is slipped into one of the cut veins, and the blood allowed to enter the jar. (If the throat is cut completely, as is usual, the operation becomes unnecessarily bloody and filthy.) As soon as the jar is filled, the tube is withdrawn and the cotton stopper readjusted. If no more blood is to be obtained, the tube is now washed well in a normal salt solution to prevent the clotting of adhering blood upon the surface and in the interior. The full jar is set aside for fifteen or twenty minutes for the clot to form; when this has occurred, a long, stiff piece of wire is sterilized in the flame and inserted into one of

the openings in the cover of the jar and swept about the sides of the interior so as to break the clot away from the glass, and thus permit its complete shrinkage. The wire is then withdrawn and the opening closed with its cotton plug. Thereafter the jar should be transferred to the laboratory with as little disturbance of its contents as possible and placed in the refrigerator for twenty-four to forty-eight hours for thorough separation of the serum from the clot. In the mean time a series of large test-tubes or narrow flasks of suitable size are arranged for receiving the serum and sedimentation of the blood-cells which are apt to be transferred with it (Fig. 28). In such a series of sedimentation tubes the connecting glass tubes are set into double-perforated rubber stoppers. All the parts are first well cleaned, the stoppers loosely

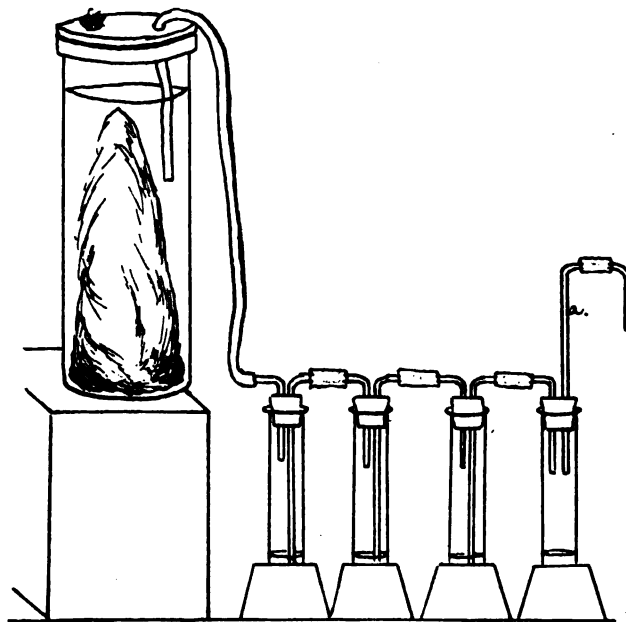


FIG. 29.—BLOOD TUBE WITH SIPHON ARRANGED TO TRANSFER SERUM FROM JAR TO SEDIMENTATION TUBES; TUBE *a* TO BE KEPT ABOVE LEVEL OF SERUM IN LAST TUBE.

applied, and the series folded together in compact manner and sterilized in the autoclave. On removal from the autoclave the stoppers are at once firmly adjusted and the series of tubes placed upon supports made of blocks of wood with suitable holes bored into them. A sterilized rubber tube is now inserted through one of the openings in the jar, well down into the serum overlying the clot, and connected with the first tube of the series. After this connection is made a second rubber tube is connected with the escape of the last of the series to serve as a mouthpiece, and strong suction made to start siphonage into the test-tubes. If the apparatus has been arranged with the jar considerably higher than the series of tubes, the siphonage will without difficulty carry the serum from one tube to another in the series and nearly fill all (Fig. 29).

white mass, like the boiled white of an egg. This step should not be hastened, and the temperature should not be allowed to go above the limit mentioned, lest the surface of the solid serum be blistered by bubbles and rendered unfit for use. After the serum is thus solidified firmly it is transferred to the steam-bath and sterilized in streaming steam by the fractional method.

A fault in the product of both the old and the present methods of preparation is due to the formation of a dense film on the surface of the serum, from drying, within a comparatively short time. To obviate this it may be recommended that a drop of glycerine should be placed in each tube of the liquid serum before it is subjected to coagulation; or it may be prevented by covering the mouth of the tube with a rubber cap or similar device.

Exercise 29.—Each student, having previously prepared a pipette (fifty cubic centimeters) by placing a cotton stopper in the upper end and then sterilizing in the autoclave, should withdraw fifty cubic centimeters of serum from the sedimentation jar, where it has previously been collected from the blood, before the class and distribute it to one dozen culture tubes. These are then arranged in proper slanting position in a wire cage and placed in the dry-air oven, where they are to be heated until coagulated firmly. Heating should be cautiously done, the temperature raised slowly to 80°–85° C. and maintained at this point until the coagulation is definitely started, when it is further increased to 90° C. for ten minutes longer. It will probably require from the first about half to three-quarters of an hour for completion of the coagulation, and close watch should be maintained upon the thermometer of the oven during the time. Overheating will cause failure from blistering the surface of the serum, such tubes being of no further service. After the serum is set the tubes are to be transferred to the steam sterilizer, the top of which is loosely applied, and steamed for twenty minutes on three successive days.

Loeffler's Glucose-bouillon-serum.—For its particular value in the cultivation of the organism of diphtheria this modification is suggested by Loeffler and is of popular use. Three parts of liquid serum are mixed with one part of sterile glucose bouillon (preferably, the latter should be made from veal). The subsequent steps of preparation are the same as outlined for ordinary serum.

6. Milk.—Aside from its value as an ordinary culture medium, milk is made use of in bacteriologic work in determination of the production of rennet-forming ferments by bacteria, as well as in the study of acid formation. The elements which are of principal nutritive value to the bacteria in milk are its proteids, milk-sugar, and the salines. For such use the milk should be obtained as fresh as possible and should be freed from fat. This last may be done by allowing the cream to separate spontaneously from the milk in a sterile jar in the refrigerator and then siphoning or pipetting the milk from beneath the layer of cream into the culture tubes; or, as is more convenient in most laboratories, fresh separator milk from the dairy may be employed and at once distributed to the tubes. The reaction of the milk should first

have been determined, although if it prove not above +2 it is not customary to make any closer adjustment to the standard; if, however, it be more acid than this, the proper correction should have been made before the milk is distributed to the tubes. Having been tubed, it is then sterilized in the steam-bath at 100° C. by the usual fractional method.

Litmus milk is prepared by adding to the above sterilized tubes, after faintly alkalinizing the milk with sodium hydroxide solution, sufficient sterile litmus solution to give a distinct but pale blue tinge to the milk. If added prior to the sterilization, the blue color of the litmus milk is liable to be changed to a dirty reddish-brown color. The preparation is used in the study of acid production.

PRESERVATION OF MEDIA AFTER PREPARATION.

In order to prevent drying of the media in tubes, which is likely to produce a tough scum upon the surface unfavorable for the development of bacteria, and may cause much shrinkage of the mass, it is well to close the mouths of the tubes over the cotton stoppers with rubber caps, rubber stoppers, tinfoil, or some other device. Before capping a tube the protruding portion of the cotton stopper should be trimmed off with a scissors and the surface of the cut end of the stopper, as well as the lip of the tube, flamed to destroy any infection which may possibly persist. The cap, or whatever else is employed as a cover, should have been disinfected in a carbolized solution and rinsed in well-boiled water before application. Should these precautions be omitted or the stopper not have been originally thoroughly sterilized, the moisture evaporating from the medium in the tube is likely to collect in the stopper and favor growth of any infectious elements which may not have been destroyed. Properly applied, however, a rubber stopper or cap over the cotton plug is of much advantage in the preservation of the medium.

The same end may be attained by keeping the tubes of nutrient substance in covered jars; these should be well sterilized before the tubes are placed in them. Moreover, the tubes should be transferred to the jars directly from the sterilizer, before mould-spores or bacteria have chanced to come in contact with the cotton stoppers; otherwise the moisture sure to be retained in the cotton will favor the most profuse growth of contaminating organisms in the plugs and these will eventually penetrate the latter and gain entrance to the nutrient substance in the tubes. If a small piece of gum camphor be placed in such a jar, it will be found of considerable service in checking the development of moulds, the small amount of camphor absorbed by the medium from the air of the jar not being of consequence usually.

In summer weather, especially in southern latitudes, it is best to keep gelatine tubes, properly sealed and capped, in the refrigerator, as the prevailing temperature is usually sufficient to liquefy the medium.

LESSON V.

INOCULATION OF MEDIA AND CULTIVATION OF BACTERIA.

For convenience of description it is supposed that the student in the work of this and the following two lessons is dealing with material containing but a single form of microorganism and that the cultures obtained are pure, although in practice this can scarcely be expected to be realized.

APPLIANCES FOR AND METHODS OF INOCULATION.

The Platinum Needle.—There is no other appliance so constantly used in bacteriologic technique as the platinum needle. It is made by fusing into one end of a glass rod the end of a bit of heavy (about No. 27) platinum wire, five or six centimeters

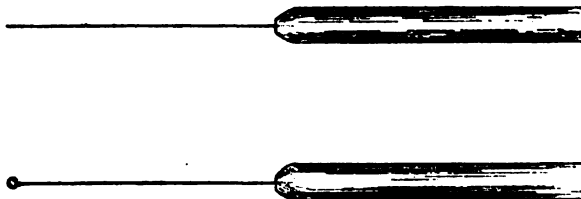


FIG. 32.—STRAIGHT PLATINUM WIRE AND PLATINUM WIRE LOOP.

in length. Platinum and glass are employed for no other reason than that they lend themselves readily to sterilization in the flame without danger of destruction. Any sort of wire, as iron or copper, with or without a handle, may be substituted should necessity demand such extemporization. A number of special shapes have been suggested for the needle, but with a plain, straight wire and one twisted into a small loop at the end (known as the "Oese" or "loop"), one may proceed without difficulty. The loop is generally selected when a liquid material containing microorganisms is to be conveyed to the nutrient medium, or when the substance to be inoculated upon the medium contains relatively few bacteria; it is used mainly for surface inoculations upon the solid media and in inoculating liquid media. The plain needle is used when the matter to be transferred is rich in bacteria, as in inoculating from an old culture on solid medium to a fresh tube, and in making stroke inoculations and puncture or stab inoculations. If bent at right angles to itself at the very end of the wire this last needle is especially suited for picking out some special growth from a mixture of colonies in an impure culture in order to transfer it to a fresh tube for the production of a pure culture. In every use of the needle (Fig. 32) it must never be forgotten

that it is absolutely necessary, in order to succeed and in order to avoid danger, that the needle should be thoroughly sterilized in the flame (*v.* exercise 6), both before taking up the infectious matter and after inoculation is completed.

If it is desired to transfer some growth from one tube to a tube of fresh solid medium, the two tubes are grasped in the left hand as shown in the accompanying figure (Fig. 33), the upper ends of the tubes being held well over the ulnar edge of the palm. The cotton plugs may then be removed with a pair of dissecting forceps and laid upon a fresh piece of paper (or may have been removed by being grasped between the fingers of the left hand before the tubes were placed in position, the loose ends being held between the fingers and the deeper ends projecting beyond the dorsal surface of the fingers). The tubes should be held in a horizontal position or slightly inverted so as to prevent as much as possible the entrance of chance organisms from the atmosphere; this position is not possible if one or other of the tubes contains liquid material, in which case the tubes must be kept upright. After the stoppers have been disposed of and the tubes properly held, the needle is taken in the right hand, held as one would

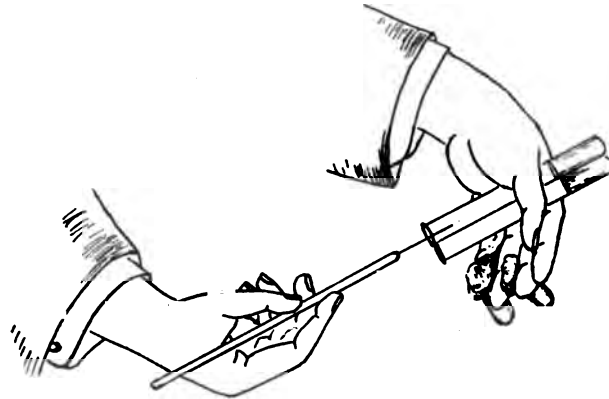


FIG. 33.—PROPER MODE OF HOLDING TUBES OF SOLID MEDIUM IN INOCULATION.

hold a pen, and flamed until the whole length of the wire has taken on a cherry-red color, and the lower end of the glass handle has been exposed to the flame. For a few moments it is allowed to cool so that its heat will not prove fatal to the germs with which it is to be brought in contact (*v.* Fig. 2). As soon as safe the needle is thrust into the infected tube and brought in light contact with the culture. Even though one cannot see it, many of the individual germs adhere to the wire. It is at once withdrawn and carried quickly and steadily into the second tube. The needle may now be drawn in an even line over the surface of the medium, the growth which develops appearing as a linear, more or less continuous colony, known as a "*stroke*" culture; or it may be rubbed irregularly over the surface, when it gives rise to an irregular film or to isolated growths, which is spoken of as a "*smear*" culture; or the plain, straight needle may be thrust evenly down into the mass of the medium, when the growth following the track of the puncture is spoken of as a "*puncture*" or "*stab*" culture (Fig. 34). The loop is especially adapted for making a smear culture. If the medium to be inoculated be liquid, the needle is merely agitated slightly in it in order to set free some of the adherent organisms in the liquid.

As soon as the inoculation is accomplished the needle is to be withdrawn carefully from the tube and flamed before being laid down; if it be wet from some liquid medium with which it has been brought in contact, it should be held for a moment by the side of the flame in order to dry before being thrust directly into the flame, as the bubbles bursting in the flame might scatter the infection and cause harm. The needle having been disposed of, the mouths of the two tubes are held for a moment in the flame to destroy any infection which may be adherent to the lip and might be forced into the interior in the application of the stoppers; and the stoppers are taken up one after the other with a pair of forceps, flamed, and thrust into the tubes. (Do not mix the stoppers.) The newly inoculated tube is then properly marked and set aside for development.

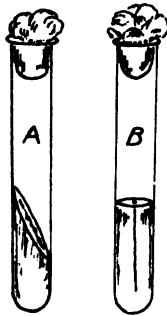


FIG. 34.—A. STROKE CULTURE. B. PUNCTURE CULTURE.

The manipulation in using the platinum needle is practically the same as the above from whatever source the infectious material is to be obtained or whether the inoculation is to be made upon media in tubes, dishes, on plates, or in flasks. The needle is first sterilized, then brought in contact with the infection, carried into contact with the fresh medium, and either drawn over its surface in a stroke or a smear or thrust into the mass as a stab (or agitated in liquid media), withdrawn from the medium, and flamed; the lip of the tube or flask flamed; the stopper flamed and readjusted (or the cover of the dish reapplied).

Swabs.—Sterile cotton swabs are often convenient for obtaining the infectious matter for inoculation, as from diphtheritic sore throats or the surface of a wound. Usually a number of such sterile swabs are kept ready in laboratories, inclosed in sterilized test-tubes. The tubes should be at least seven inches in length and preferably three-fourths of an inch in diameter. A piece of ordinary brass or iron wire is cut a little shorter than the tube and one end is roughened with a file. About this end a firm swab of absorbent cotton is twisted, and its surfaces rolled smooth between the fingers. The opposite end is set into a small cork and absorbent cotton is firmly wrapped about it, completely covering in the cork, until the mass will fit closely, but not tightly, in the tube. The tube having been cleaned in the usual manner, the swab is introduced into its interior, the cotton plug wrapped about the handle of the wire serving as a stopper for the tube. The whole appliance is now thoroughly sterilized in the autoclave and thereafter a rubber cap is applied over the mouth of the tube to protect against microbic penetration and to keep the swab moderately moist. The tube with the contained swab may be carried to the bedside, where the swab is withdrawn, brushed over the infected surface, and at once replaced in the tube and carried to the laboratory. Here the swab is used just as an inoculated platinum needle would be used for the making of smear inoculations upon the surface of media in tubes or dishes or on plates. After the inoculation has been

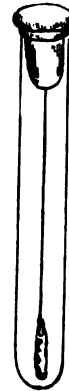


FIG. 35.—STERILE COTTON SWAB IN TUBE, WITH RUBBER CAP OVER MOUTH OF LATTER FOR GREATER PROTECTION OF SWAB AND TO PREVENT DRYING.

performed, if there be no further need for the material, the swab is burned and the tube disinfected and washed in the usual manner (Fig. 35).

Pipettes.—Ordinary graduated pipettes of small caliber are often used for making inoculations with definite quantities of liquid containing infectious germs, and simple pipettes drawn in the laboratory from narrow glass tubing are likewise used for the transfer of liquids to the nutrient media. The liquid thus added is usually diffused through the nutrient which has been previously liquefied by heat, and the contained microorganisms are thereby scattered throughout the nutrient mass. The resultant culture thus grows either diffusely or in separate colonies (each representing at least one bacterium) throughout the medium; such a culture is spoken of as a "*diffusion culture*." In practice, the pipette, the upper end having been provided with a cotton stopper, is sterilized in the usual manner in the oven or autoclave. If desired, a piece of rubber tube may be attached to the upper end to serve as a mouthpiece. The

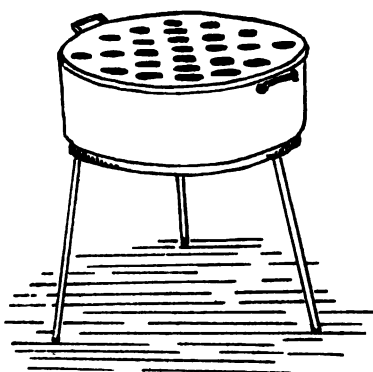


FIG. 36. — LABORATORY WATER-BATH FOR MELTING SOLID MEDIA IN TUBES.

small end is passed several times through the flame as a precautionary measure and then plunged into the infected liquid, which is drawn into the interior to the desired graduation by suction by the mouth. A tube of liquid medium is at hand or a gelatine or agar tube is liquefied in a water-bath (either one extemporized or of a form especially designed for the purpose, as shown in Fig. 36). The stopper is removed from the culture tube and the measured quantity of the infected liquid passed into it. The pipette is at once laid aside (into a pan of disinfectant solution, if of no more consequence), the lip of the tube flamed, and the stopper flamed and readjusted. Then, with slight agitation, care being exercised that the stopper is not soiled by the contents of the tube, the material is thoroughly diffused through the medium. The tube may now be set aside

and the contents allowed to set, or the latter may be poured into a Petri dish or upon a plate for a dish or plate culture, if so desired.

Should the liquid to be inoculated upon the nutrient medium contain but few organisms, the usual amount employed is one cubic centimeter, which amount is generally diffused in nine cubic centimeters of the medium. If there be numerous organisms, however, so that in the resulting culture the colonies are likely to be so numerous and grow so close to each other as to interfere with proper observation and enumeration, known dilutions with sterilized water may be made to reduce the number of bacteria in the volume employed—as one part of the suspected fluid to nine, or ninety-nine, or nine hundred and ninety-nine parts of sterilized water. When very small fractions of a cubic centimeter of the original liquid or of the known dilutions are desired, they are best obtained by means of *long capillary pipettes* drawn from narrow, soft glass tubing as needed. (To draw such a pipette select an eight- or ten-inch piece of a soft glass tube of small caliber and heat a length of several inches near the middle in the spread flame until it has become ductile; then at once remove from the flame, and with even, fairly rapid traction from each end draw the tube into a uniform

capillary diameter and extent of several feet. As soon as cool the tube is cut or broken so that as much as possible of a uniform caliber of the capillary portion remains attached to one end of the original glass tube.) Into this capillary is drawn a known quantity of water (as 0.5 cubic centimeter) from a watch-crystal into which it has been carefully measured. The length of the column of water in the capillary is marked with a pen, and convenient divisions for fractions of the contents are similarly marked at proper intervals upon the glass. The water is now blown out, the tube washed with alcohol and ether to insure dryness, and then sterilized in any large oven in the usual manner, a plug of cotton having been previously placed in the larger end. (In the absence of oven large enough to accommodate a long pipette, it may be sterilized by drawing it full of a disinfectant solution and immersing its capillary end in the disinfectant, and thereafter washing out the disinfectant with well-boiled water, and rinsing with alcohol and ether.) Thus prepared it is to be used in the same manner as an ordinary pipette in inoculation exercises.

Syringes.—For collection of liquid material and its convection to the nutrient material from a living diseased individual, as splenic blood, blood from a vein, the contents of a cyst or of an abscess or blister, syringes are often used. These should be of a form and construction easy of sterilization, as Koch's inoculation syringe (Fig. 37). All parts of the syringe having been sterilized (glass and metal portions in autoclave or oven, rubber parts in disinfectant solution and boiled water) and adjusted,

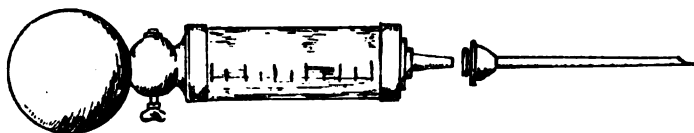


FIG. 37.—KOCH'S INOCULATION SYRINGE.

the needle is thrust into the tissues to the source of the desired material and the liquid drawn into the syringe. (Any part of an exposed surface to be punctured should have been previously sterilized in surgical fashion.) Observing the usual precautions, the needle, after withdrawal from the tissue, is introduced into the culture tube, a drop or more of the contents expressed upon the nutrient medium, the syringe disposed of, the tube closed with the usual precautions, and the added matter diffused in the medium by gentle agitation. The syringe is then to be cleaned and sterilized and its contents destroyed.

A very convenient substitute for a syringe is the *Sternberg bulb* (Fig. 38), which may be made in a few minutes from a piece of soft glass tubing. (One end of a piece of glass tube about eight or ten inches in length is fused shut in the Bunsen flame and about an inch of the closed end softened, from which a bulb is blown by the operator to half an inch or an inch in diameter. After having become cool, the tube, as close to the bulb as possible, is softened in a narrow flame for a distance of half an inch or an inch and drawn to a fine capillary end, which if desired may be sealed shut in the flame.) If sealed in its manufacture, the air in the bulb will be much rarefied and the interior of the appliance may be regarded as having been sterilized by the heat applied during manufacture. As a uniform precaution, however, it is well to sterilize the bulbs, whether sealed or not, in a metal box or wrapped in paper, in the oven, and the protection of the box or cover retained until the bulb is to be used. In use, the air in the

interior of the bulb is forced out by holding it close to a flame, and as soon as this is done the capillary end of the tube is plunged into the liquid, the latter being then forced into the interior by external air pressure. After the liquid has entered the bulb the capillary end may be sealed and the bulb and its contents carried to the laboratory for use in inoculation. (If the capillary end was sealed before the collection of the liquid it should have been broken off with a sterile pair of forceps, preferably beneath the surface of the liquid. If the contents of a blister were to have been collected, the surface of the lesion should first have been sterilized surgically and a puncture made with a sterile blade through which the capillary tube is inserted to the interior of the sac.) In the laboratory a tube of medium is placed in a water-bath for liquefaction, and the capillary tube of the Sternberg bulb disinfected and rinsed in boiled water. The plug is withdrawn from the culture tube in the usual manner, and the capillary of the bulb inserted, the sealed end having been broken off with a sterile forceps. The close application of the palm of the hand to the bulb will generally give sufficient warmth to force the contents into the culture tube drop by drop; or a flame may be brought close for the same purpose. After one or more drops have been transferred, the bulb is disposed of, the tube closed in the usual fashion, the infectious material diffused through the medium by agitation, and the tube set aside for whatever purpose desired. If there be no further need of the bulb and its contents, it should be placed in a disinfecting solution and subsequently destroyed in the furnace.



FIG. 38.—STERNBERG BULB.

Knife-blades.—In the course of autopsies or surgical operations scrapings from the tissues in suspected foci may be transferred to the nutrient media by means of long, slender, sterile knife-blades. Thus, if an enlarged gland is to be submitted to a culture examination, such a blade (sterilized in the autoclave or by boiling or disinfectant solution—or, if not valuable, by flaming) is driven with its cutting edge well into the tissue, turned on edge, and withdrawn so that the edge will scrape some of the pulp off the cut surface. A tube of medium is taken up and opened as usual, the blade inserted, and some of the adherent pulp smeared well into the surface of the medium if solid, or the blade waved gently in the medium if the latter be liquid, so as to dislodge into it some of the pulp. The blade is then withdrawn and if of no further service is placed in a dish of disinfectant solution. The lip of the tube is flamed, the stopper flamed and adjusted, and the tube, properly marked, is set aside for development of the inoculated bacteria.

Forceps.—Long, slender, sterilized forceps are sometimes used for the conveyance of infected solid substances to the culture medium.

Particles.—Under various circumstances small bits of solid substances which have been in contact with the infectious matter may advantageously be themselves transferred, with the microorganisms adhering to them, to the nutrient material. For example, when the upper end of the culture tube has been drawn shut in the flame, as is occasionally done instead of closing with a cotton plug, it is a matter of some difficulty to arrange for the entrance of the platinum needle. In such a case it is possible to take up a *bit of platinum wire* in a forceps and sterilize it well in the flame, dip it into the infected substance, and then drop it into the tube through a small opening made by breaking off the tip of the sealed end. The opening thus made is then again sealed in the flame and the tube agitated so as to diffuse the bacteria on the wire through the liquid medium, or over the surface if the medium be solid. Some-

times, when a comparatively pure water is to be examined, the bacteria from a large amount are concentrated by filtration through sterile powder, and *particles of the powder* are transferred to the culture tube by means of the forceps, carrying with them a greater or smaller number of the germs. *Short lengths of capillary tube* (sterile), into which a suspected liquid has been drawn, may be broken off and thrown into a tube of liquefied material and the inclosed liquid diffused through the medium by agitation; the known length of the tube affording opportunity of approximate quantitative estimation of the organisms developing into colonies in the medium if desired. Another and somewhat similar method of approximate quantitative estimation of bacteria in a liquid is to take equal lengths of *thread* and place one in a liquid of the same general nature as that to be examined but not infected (water or bouillon), and a second into the fluid to be investigated. The first is weighed when wet and placed in the oven to dry, after which it is again weighed, the difference in weight representing the fluid it was capable of absorbing. The second piece of thread is taken from the infected fluid with a sterile pair of forceps, and cut with sterile scissors at a definite length (one-half, third, or fourth, as desired), and the bit thus cut off transferred to the tube of liquefied medium, and the tube agitated so as to scatter the bacteria from it throughout the medium. Thereafter the tube is set aside for growth as a diffusion culture or its contents transferred to a Petri dish or plate as best suited. *Sterile granulated sugar* is often used in filtration of air and then placed in liquefied medium so that the sugar may dissolve and leave the bacteria which had collected in the filter upon the grains scattered through the medium.

Fractional Inoculation.—When the substance to be inoculated into a nutrient material is especially rich in microorganisms, the resulting cultures are apt to be so crowded with colonies, of single or mixed type, that it is impossible to recognize the characteristic appearances of a single colony or to separate it from the mass. To prevent this, *dilution inoculations* are generally made. A series of three culture tubes is usually used, the first to be inoculated from the original infected matter, the second from the first tube, and the third from the second. Thus, for example, in the clinical cultivation of the diphtheritic organism, *Mycobacterium diphtheria*, three tubes of blood-serum are usually inoculated. The first is inoculated by smearing the swab, infected from the patient's throat, over the surface of the solidified serum. The platinum loop is then taken up and sterilized and drawn over this inoculated surface, and then rubbed over the surface of the serum in the second tube. The loop is again flamed and drawn over the surface of the medium in the second tube and rubbed over that in the third tube, probably carrying to this last but few of the organisms which were originally deposited upon the serum of the first inoculation. On comparison of the three tubes after growth of the infections the value of the procedure will at once be appreciated, the scattered colonies in the third tube being much the most easily studied and the most characteristic in appearance.

The same principle is followed in diffusion inoculations of material rich in bacteria. The first tube having been inoculated and the material diffused in the liquefied medium, a given small amount (as one, two, or three loopfuls, or more definite amounts, as a fraction of a cubic centimeter, if desired) of the mixture is carried into the second tube and similarly diffused. The procedure is repeated from the second to a third tube, in which the organisms of this final dilution are likely to be few and scattered and the colonies resulting from their growth distinct and characteristic.

SOURCE AND COLLECTION OF MATERIAL FOR INOCULATION.

The wide range of parasitic and saprophytic occurrence of bacteria renders it impossible to indicate any methodical procedure in their collection for inoculation, nearly every case requiring some individual detail and particular precaution to insure success. The physical character of the substance to be examined (whether gaseous, liquid, or solid), its richness or poverty in bacteria, the simplicity or complexity of the bacterial flora, and a variety of other considerations all influence the plan of procedure in a greater or less degree.

In whatever manner the material be obtained or whatever its source, as soon as it is removed from its natural surroundings and conditions, it is to be kept until actual inoculation is accomplished in perfectly sterile containers, contact with every unsterilized object affording opportunity for further contamination and invalidating the results of the study. So, too, the least possible delay in inoculation should be permitted after the acquirement of the infected material, especially if estimation of the number of bacteria, as well as of their types, is to be included in the study; since the chance for numerical increase or loss or even of loss of types in a comparatively short period of time after their removal from their natural relations can easily be verified. For example, a water containing organic contamination may, owing to a prevailing low temperature, be infected by comparatively few bacteria when collected; yet, if kept for but a few hours at the higher temperature of the room, before inoculation this number may enormously multiply and the result represent a condition of contamination many times that actually existing in the water in nature. Moreover, owing to different rate of growth, one type represented by few individuals in the original sample may in the room temperature come to an exaggerated proportion as compared with another; or the latter might actually be crowded out of existence by the antagonistic influences of the first.

In a general way it may be said that where the infected material is rich in bacteria and its flora varied it is advisable to collect small quantities and to further isolate the bacteria by dilution with sterile diluents, either before or during the process of inoculation; and that, on the other hand, when a substance is poor in its bacterial flora, in order to more certainly obtain all the types of its scattered organisms it is essential that by filtration or some other method concentration of the organisms should be accomplished before inoculation. Thus, sewage water, crowded with microbic life, must for convenience and for reliability of results be diluted with sterile water (or dilution inoculation performed), often to an extreme degree; while a pure potable water should be passed in large amounts through a suitable filter so as to collect in the latter its occasional, but perhaps variant, types in a convenient compass. The organisms of the air are likewise usually collected by filtration on sterile filters. The uncertainty of contact of the bacteria in a solid substance with the nutrient material upon which the solid may have been inoculated usually makes it advisable that such solids should as a preliminary measure be well diffused in sterile water, portions of which are thereafter transferred to the medium, with the result of more certainly and directly implanting the bacteria upon the nutrient.

In obtaining the infected material from the living body, probably more than under any other circumstances, the necessity for precaution to prevent contamination by bacteria not concerned in the disease under investigation will be appreciated. The presence of an enormous variety of natural but unimportant organisms in the exposed parts of the body, the difficulties of manipulation, the uncertainties in selective

operation, all combine to render the discovery of pathogenic germs a most difficult part of bacteriologic investigation.

Briefly stated, it is to be kept in mind, as of fundamental importance, that the material collected should be obtained *so as to represent its complete natural flora*, that it should be *preserved from contamination and intrinsic changes* before inoculation or *at once inoculated* upon the nutrient medium, and that the infectious elements collected *be diluted or concentrated* by artificial device as required for convenience of manipulation and certainty of investigation. For these desiderata the application of method, care of procedure, and a variety of needed apparatus must call forth the ingenuity and dexterity of the student; and each case is to be approached individually for the application of these principles, so as to provide eventual success. It is impossible to particularly describe the technique of special procedures, and the consideration of a few general examples, such as will presently be outlined (air, water, milk, soil, and disease material), must serve as illustration.

THE DIFFERENT FORMS OF CULTURES.

Tube Cultures.—Growths of organisms inoculated upon media in test-tubes are least liable to contamination from the various possible accidents of manipulation, and are the most conveniently prepared and handled; they are therefore the type usually employed unless some special purpose demands the use of others. Cultures in tubes of liquid media are, of course, *diffusion cultures*, the organisms readily spreading through the liquid in which they are grown. On solids, the cultures, from the mode of inoculation and from peculiarities of the bacteria themselves, may be limited to the surface (*surface cultures*), as *smear* or *stroke cultures*, or may grow in the interior of the mass, as *stab cultures* or *diffusion cultures*. The same microorganism grown by these different modes even upon the same medium is likely to present considerable differences of appearances and of rate and profusion of development. Diffusions and smears usually show the most characteristic appearances, although both punctures and stroke cultures afford important information. Any of the media may be used in tube cultures.

Plate Cultures.—These were introduced by Koch for use in the separation of mixed into pure cultures, and although largely superseded by Petri dish cultures and Esmarch's tubes, are still frequently employed. In preparing a plate culture, the glass plates, supports, and culture dish already described (*v. Apparatus*, p. 64), sterilized as indicated, are to be provided, three plates and their platforms being usually arranged as a set in a culture dish. Three diffusion inoculations are generally made of the material to be examined; the first from the original material, the second by the transfer of a small amount (one or two loopfuls) of the first diffusion to the medium in the second tube, and the third by like transfer from the second to the third tube. (Sterilize the loop between inoculations.) A level surface is provided, usually by means of a platform tripod with adjustable legs (Fig. 39), upon which is placed a flat dish filled level full with ice-water or crushed ice. This dish is covered with a large glass plate upon which rests a glass bell jar. The glass plate and bell jar should have been disinfected and rinsed in boiled water before use, or otherwise sterilized. One of the plates intended for the culture is placed upon the chilled surface of the glass plate and the liquefied medium of the first diffusion inoculation poured over it, the mouth of the tube having been flamed before the medium is poured out. The bell jar is at once lowered, and by tilting the glass cover of the ice dish the liquid medium can usually without difficulty be made to cover the culture plate in a fairly even film.

Should there be difficulty, however, a glass rod is quickly flamed and introduced under the bell jar and used to spread the medium before it sets. One of the sterilized platforms is now placed in the culture dish and the culture plate adjusted upon it as soon as the film of medium has become solid, the cover of the dish being immediately reapplied. The same process follows in turn for the second and third plates, upon which are spread the contents of the second and third diffusion tubes, the plates being arranged with their platforms in a set, one over the other in the culture dish. In work of this sort gelatine is usually used as the medium, being more liquid at temperatures which are harmless to the implanted microbes than agar, which at the same temperature is less easily spread and apt to quickly solidify in an uneven layer where it is deposited on the plate. Gelatine-agar may, however, be employed, and is preferable to plain gelatine in that the dish and plates may be introduced into the incubator without danger of the medium being liquefied and running off the plates.

Plates prepared in the same way with uninoculated medium are sometimes used for smear or stroke inoculation when it is wished to have a large surface for distribution of the infectious material. For this purpose well-liquefied agar may be employed as well as gelatine.

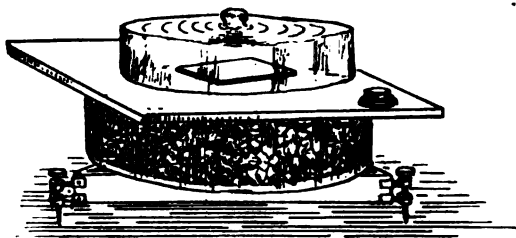


FIG. 39.—LEVELLING TRIPOD BEARING A DISH OF CRACKED ICE, COVERED BY A GLASS PLATE. ON THE PLATE A LEVEL AND BELL JAR; BENEATH THE LATTER A CULTURE PLATE.

As a result of spreading the diffusely inoculated medium over the extensive surface of the culture plate, the colonies arising from the multiplication of the individual microbes are usually scattered and isolated from each other, thus affording facility for counting their number and for examination of their peculiarities of form and color, as well as favoring any attempt at mechanical removal of one or other colony to a fresh medium in order to obtain a pure culture. With a magnifying glass or the low power of the microscope one can frequently appreciate peculiarities of importance in the identification of the organisms, which might not have been possible if the colony were in the ordinary culture tube. These advantages have made the principle of plate cultures of the utmost value to bacteriologists. "To plate a tube" is an expression often used in the laboratory, by which is meant the spreading of the contents of a tube over a broad surface, whether on plates, as above described, or in Petri dishes, or over the interior surface of the tube, as in Esmarch tubes.

Petri Dish Cultures.—The objectionable features of the ordinary plate culture are the danger of infection in preparation (common to all cultures in large culture dishes), the rather tedious technique of spreading and solidifying the medium over a cold surface, the possibility of the liquefaction of the medium during culture and dripping from the plate, and the difficulty of examination of the colonies without

taking the plate out of the culture dish and thus exposing it to atmospheric contamination. To offset these objections the small flat dishes known as Petri dishes have been introduced. They are used for precisely the same purposes as the plates and may be thought of as individually covered plates. In their employment the same manipulations are to be practised as in case of the preparation of plate cultures; but there is not the same need of a perfectly level surface in spreading the medium over the bottom of such a dish, nor is it requisite that the medium should be immediately solidified by cold, as is done in case of plate cultures, to prevent the liquid medium from flowing over the edges of the plate. The only objectionable feature in their use—a very real objection, but not nearly so serious as when sets of plates are being arranged in a large culture dish—is the chance of entrance of contaminating organisms from the air when the lid of the dish is raised for the introduction of the liquefied medium. From imperfect fitting of the lid to the dish, particularly in careless handling, there is some danger of entrance of bacteria from the outside; but, as already suggested, this may be largely obviated by sealing the edge of the cover over the dish by a strip of paper or rubber band or other device.

Esmarch's Tubes (Rolled Tubes).—As a further development of the same principle Esmarch suggested the absence of need of exposure of the medium, either in dish or on plate, if the liquefied material after inoculation be spread over the interior surface of the tube itself and solidified in this position. This idea leaves little to be desired if the amount of the medium and the size of the tube be properly proportioned. For reasons before mentioned gelatine lends itself better than the other media for the purpose involved. Obviously, however, tubes of this form in which gelatine is used as the medium should not be exposed to temperatures above 25° to 28° C., lest the gelatine be liquefied. Rolled tubes of gelatine-agar or of plain agar may, however, be incubated without danger. In preparing a rolled tube a block of ice should be obtained, and its upper surface cut nearly, but not quite, level. A small groove should be cut in the ice, leading from the higher edge over the slightly sloping surface and its irregularities melted away by applying a tube of hot water in the groove. The tube of liquefied gelatine is now inoculated, the infectious matter well diffused by agitation, and it is then laid in the groove on the ice block, care being taken that the gelatine does not come too close to the stopper. Here it is steadily rolled around in the groove until the whole interior surface from the bottom to within about half an inch of the stopper is evenly coated with a film of the gelatine and the latter has become solid. The same end may be accomplished by twisting the tube held in a nearly horizontal position under a tap of cold water, but the first method is more satisfactory.

In such a tube all the advantages of isolation of the colonies for transfer to fresh media, and for enumeration or examination, exist as in case of plates or Petri dishes, and the procedure is to be strongly commended.

Flasks.—What has been said of tubes applies to flasks, with whatever difference the size of the latter may occasion. The flask cultures are only used when large quantities of some isolated species are desired.

ILLUSTRATIVE PROCEDURES.

Examination of Air.—The mere fact of the presence of microbic life in the atmosphere is almost axiomatic, and has been illustrated by the experiences gained in exercise 5 or 23, or by the decomposition of media or of organic substances used in our daily

life by infection of these when accidentally or carelessly exposed to the air. (In the last connection, however, and in the transmission of disease to individuals the rôle of flies and other insects as conveyers of the infections must be kept in mind.) Both vegetative and spore forms of bacteria are included in the atmospheric flora. For the most part they are attached to particles of dust or of moisture in the air, and for this reason are more numerous in the lower than in the upper strata of the atmosphere, and in dusty or moist air rather than in a clean and dry air. It is to be presumed that unless exceptionally they do not develop in the air, but are conveyed by currents from some point of origin; for which reason they are more common in the atmosphere of populous districts, where there is abundant opportunity for the development of parasitic and saprophytic germs, than in that of dry, barren, unpopulated areas of land or in the air over the ocean far from the shore; and for the same reason, in the atmosphere of warm, moist localities and in the summer, rather than in cold, dry climates. So, too, one may expect to meet in the air of any selected locality more bacteria when there are strong currents than when it is still. Cornet has estimated the existence of over three hundred bacteria in a cubic meter of the air passing over the house-tops of the city of Paris; and thousands may be demonstrated in the same volume of air from the gutters and dirty cellars of cities.

For collecting and cultivating these atmospheric organisms a variety of devices

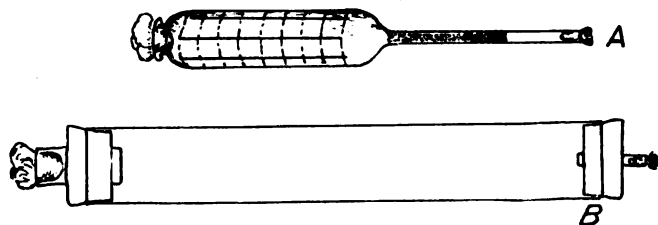


FIG. 40.

A. Sedgwick-Turner aero-bioscope. B. Glass tube of Hesse's apparatus.

have been suggested, of which the following two may be described as illustrative of the principles of most:

Sedgwick-Turner Aero-bioscope.—This appliance (Fig. 40 A), as furnished in the market, consists of a glass tube of the shape shown in the diagram, and is about thirty-five centimeters in total length. The wide part of the tube is fifteen centimeters in length and four and a half centimeters in diameter, narrowed at its wide end to a neck two and a half centimeters in diameter; and at the opposite end continued as a narrow straight tube fifteen centimeters long and one-half centimeter in diameter. The wide part of the tube has squares marked upon its surface to facilitate counting the colonies of bacteria scattered over the inner surface when the apparatus is in use. A small roll of wire gauze is placed in the narrow tube just within the cotton stopper, extending to a mark cut in the glass one-third of the length of this narrow part from its free end. Both ends of the appliance are plugged with cotton and the whole sterilized in the oven in the usual manner. Some finely granulated sugar is now introduced into the wide end of the tube, sufficient to evenly fill the narrow tube above the gauze on which the sugar rests. This sugar is intended as a filter upon which the bacteria of the air will lodge and be retained as air is drawn through the apparatus from the

wide end toward the narrow portion. After introduction of the sugar the cotton is replaced in the wide mouth of the tube, the appliance held in vertical position (wide end up) and the sugar shaken into the narrow tube. It is now again baked for sterilization of the sugar for several hours at a temperature of 120° C. Held in the same position, it is then carried into the atmosphere to be examined, and the lower end attached to some form of suction apparatus, as a pair of siphon bottles (the capacity of which is known), the stopper removed from the upper end of the tube, and the siphonage started. After a definite amount of air has been drawn through the sugar the cotton stopper is replaced, the tube disconnected from the suction apparatus, and the tube held in a horizontal position and slightly shaken to throw the sugar back into the wide part of the tube. This done, twenty-five cubic centimeters of liquefied sterile gelatine are introduced into the expanded part of the tube by means of a bent pipette, the tube being maintained in horizontal position. In this liquid the sugar is soon dissolved, leaving the bacteria which were adherent scattered in the medium. The medium is now distributed over the inner surface of the expanded part of the apparatus in the same manner as in any rolled tube and solidified by contact with cold. It is set aside at room temperature for development of the bacteria, from each of which it is assumed a focus of growth or colony will form in due time. The colonies are eventually counted, from the number of which and the known amount of air drawn through the tube the degree of impurity of the atmosphere may be appreciated. From the tube special colonies as desired are to be transferred by means of the sterile platinum needle for further study as isolated or pure cultures, as described in a future lesson.

Hesse's Apparatus.—This consists (Fig. 40 B) of a large, straight glass tube from thirty to forty centimeters in length and from four to five centimeters in diameter. The ends of this tube should be provided with perforated rubber stoppers, into the holes of which are fitted short lengths of appropriate narrow tube (in one end the tube fitted in the stopper should have a comparatively large diameter, 1.5 to 2.5 centimeters, while that of the other end may be about 0.5 centimeter in diameter). The large glass tube is sterilized in the oven in the usual manner, the rubber stoppers and their fittings in disinfectant solution and rinsed in boiled water. As soon as ready the stoppers are fitted into the tube and sterile cotton plugs placed in the small tubes in the stoppers. A suitable quantity of sterile gelatine (twenty-five to fifty cubic centimeters) is now liquefied and introduced into the tube by means of a curved pipette, the apparatus being held in a horizontal position, and distributed over the inner surface and there solidified as in the preparation of Esmarch tubes. This done, the tube is carried to the atmosphere to be examined, and the narrow fitted tube connected with a suction apparatus (tube held in any position after gelatine is solidified), and the cotton removed from the other end. The suction apparatus is now set in action and a definite quantity of air drawn through the tube. It is supposed that the organisms in the air, in their progress from one end to the other of the tube, are likely to come in contact with the gelatine film and adhere to it. The cotton stopper is then adjusted, the suction apparatus disconnected, and the tube removed to the laboratory for development (at room temperature) of the bacteria which have been introduced. As in the method above described, the resulting colonies are counted, the ascertained number being accepted as indicating at least the same number of individual bacteria to have been present in the amount of air drawn through the tube; and special colonies are to be removed by means of the sterilized platinum needle for isolation and further study.

For less exacting examination the mere exposure of sterile media, as potatoes, gelatine, agar, or other substance, in open Petri dishes is usually sufficient; in such case, however, one should guard against the convection of organisms to the nutrient substance by flies and similar agents.

Examination of Water.—Depending largely upon the amount of organic impurities (there are some bacteria capable of living in filtered water, depending on the minute impurities and carbonic oxide absorbed from the air), the temperature, the exposure of the water to sunlight (inimical to the bacteria), and the aeration brought about by rapid and dashing currents, and other factors, the number and kind of bacteria found in a sample of water vary within a wide range. Artesian water, from its filtration through great distances of the ground, and underground water generally, must for mechanical reasons, if for no other, represent the purest types in nature. No definite numerical limit may be set for purity of water from a standpoint of potability, so many of the common forms of water bacteria being innocuous when taken in the human system, and a very few pathogenic germs, on the other hand, rendering a sample unfit for drinking purposes. In the most general sense, however, a water containing no more than one hundred bacteria to the cubic centimeter is regarded as a pure water; above one hundred to five hundred to the cubic centimeter, as doubtful; and above five hundred, as unfit for drinking use. Sewage water during summer temperatures may sometimes be found to contain 25,000,000 or more organisms to the cubic centimeter. In studying any sample of water, experience has shown that the really important bacteria—those of diseases like typhoid fever or Asiatic cholera, which are commonly believed to be transmitted through drinking-water—are very likely not to be found even though their presence is suspected, the conditions of collection and cultivation usually adopted not favoring their development in the cultures and consequent recognition. They are apt to be few in the water compared with the ordinary water bacteria; and the temperature of the room usually employed for cultures and the overwhelming growth of the water bacteria probably interfere with their best development. Should these or other bacteria, as the colon bacillus or the putrefactive bacteria, be recognized, however, even in the smallest numbers, the water-supply from which they have been obtained should without further question be condemned as unfit for use, and corrective measures insisted upon. Therefore, in the examination of a sample, even though it be impracticable to isolate and identify every one of the numerous forms of microbes likely to be encountered, every bacteriologic analysis should occupy at least these two phases of inquiry: *the number of organisms* in a definite amount of the sample (one cubic centimeter) and *the presence of pathogenic species* or such as indicate excrementitious or putrefactive contamination. Moreover, such biologic examination should be accompanied by the usual chemical determination of organic impurities, the number of bacteria becoming the more significant as the chemical analysis indicates a small amount of such contamination. It is not the inert organic matter present which is of direct moment, but rather the active life it supports in the water; not so much the number of these living things, but essentially their character, which must finally indicate the value or the noxious qualities of the supply. As far as the mere presence of the water bacteria is concerned, in one sense they must be regarded as coadjutors of man, in that they destroy the organic matter in water and aid in its purification—a fact easily realized by one who has been accustomed to the use of cistern water during warm seasons. After a rain, when there are carried quantities of organic refuse from the roof into the cistern from rotting shingles and from the surface dust blown on the roof by the wind, in a favoring

temperature an active fermentation prevails in the water for a number of days, during which time the water is apt to be turbid and foul and its surface covered with froth; in the course of three or four days, however, the activity of the process subsides and the water again becomes clear and attractive to the taste, the organic substances having been destroyed.

Great variation in the number of bacteria found in a water is certain to accompany marked differences of temperature; for which reason it is a fairer statement of the condition of the water to announce a mean result from several examinations made at different seasons, than to give out the result of a single investigation.

It is essential, too, that the transfer of the specimen to the culture medium should follow its collection as closely as possible; otherwise, owing to the favor of the quiet and warmth of the laboratory in which it is standing, there will be induced a great and rapid multiplication of the germs in the sample, vitiating the results of the analysis. Should it be impossible to at once proceed with the cultivation of the water after it has been collected, as where it must occupy some time in transit to the laboratory, it should be packed in ice to prevent this source of error.

The method of collection must vary with the relative degree of bacterial contamination. A small sample of a water rich in microorganisms will in all probability contain a full representation of the flora of the general supply; when the water is relatively pure, the same amount is very likely to represent it uncertainly both numerically and typically. In the latter instance it will be advisable to practise some measure which will insure the concentration of the flora of a large amount of the water, as upon some form of sterile filter, the washings from which may thus be taken as representative of the whole amount filtered.

Water in which currents prevail is likely to have its bacteria and other particles well disseminated through the whole bulk, and a single sample is therefore usually a fair example of the whole. When water is stagnant, however, the upper and lower strata are usually particularly rich in bacteria, the former with those in active growth near a free supply of air, the latter from sedimentation from the higher strata and with bacteria developing in the dead organic deposit and away from a free oxygen-supply. In the latter case, as in cisterns or infrequently used wells, it should be a rule to take samples from different depths,—top, middle, and bottom,—the report of results being the mean of the series. So, too, when water from a tap is to be examined, because of the tendency for similar sedimentation through the relatively quiet column of water in the ordinary house pipes down to the first free current in a neighboring main, it is the usual practice to let the water flow freely for at least a half hour before the sample is taken.

It is advantageous, if there be no reason to prevent and no basis for reasonable opinion as to whether there be large numbers or few bacteria in a sample to be examined, that a rough preliminary test be made by inoculating a tube of liquefied gelatine with one cubic centimeter of the natural water, diffusing and plating it. In two or three days one can easily determine the need for concentration of the specimen or for its dilution as there appear few or many colonies. If it be found that few or no growths follow this preliminary test, a filter is arranged, to be attached to the tap after the water has been running from it for a proper time (or to a large sterile funnel into which the water is poured if taken from some source without pipe connection). There is selected a glass or tinned iron cylinder about twenty or thirty centimeters in length and one and a half or two centimeters in the inside diameter. In the interior, as shown in the accompanying diagram (Fig. 41), the following layers are arranged:

At the lower end a small roll of fine wire gauze; above this a layer of absorbent cotton; over the latter a layer of fine sand about three or four centimeters deep; and above this, about the same thickness of a fine powder, as chalk, talcum, soapstone, pumice, porcelain, or glass. Thus arranged, the apparatus is sterilized in the oven by prolonged baking and then a small amount of sterile water is poured into it to moisten the different layers and cause the powder to become well packed. It is now attached to the tap by a sterile rubber tube (bound tightly with copper wire), the water turned

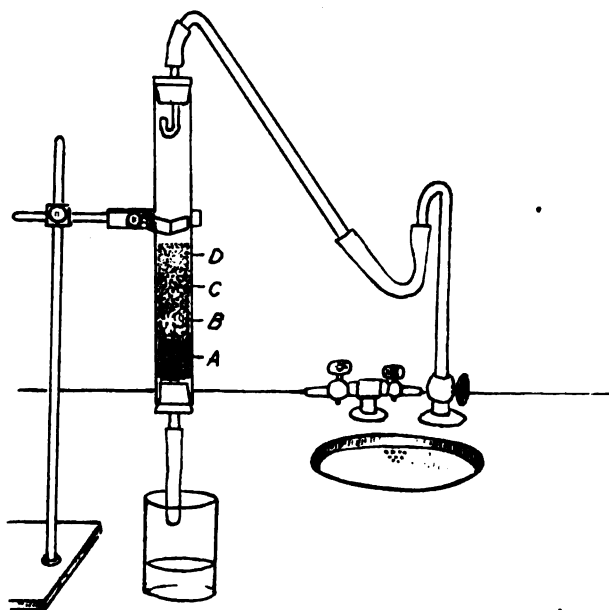


FIG. 41.—COLLECTION FILTER, FOR USE IN CONCENTRATING BACTERIA FROM A LARGE AMOUNT OF WATER.

A. Wire gauze. *B.* Cotton. *C.* Sand. *D.* Powder. Note recurved entrance tube at top of filter to prevent force of water current from disturbing the powder in filter below.

on in a gentle stream and a vessel of known capacity placed beneath by which the amount of water which has passed through the filter may be measured. After a suitable quantity has been filtered (ten or twenty gallons) the apparatus is detached and the powder and sand shaken down into a measured quantity (five hundred cubic centimeters) and well diffused in it. A known quantity of the latter, turbid with grains of sand, powder, and bacteria, is then transferred to a tube of liquefied gelatine, in which it is diffused by agitation and the medium thus inoculated is plated in one of the usual manners. After growth (usually about three days) at room temperature

the developed colonies are counted; from which number the total in the original quantity of the water passed through the filter can readily be calculated. Special colonies are removed from the medium, by means of the sterile needle, for isolation and further study.

If in the test examination the plate was crowded with growth, dilution of the sample should be practised, sterile water being added as the diluent. The sample, a small one, is collected in a sterilized glass bottle provided with glass stopper; and should it have been standing for a short time in this, it should be well shaken so as to evenly distribute the organisms contained, these having tended to settle to the bottom.

Several dilutions are generally made (1:10, 1:100, 1:1000). From each of these dilutions one cubic centimeter is transferred to a like number of tubes of liquefied gelatine, diffused by agitation, and the medium plated. As a control a small amount (0.1 cubic centimeter) of the original specimen is similarly dealt with. The inoculated preparations are then put aside at room temperature and the bacteria allowed to grow. At the end of the third or fourth day development will probably be complete (daily observation should, of course, have been made), the colonies counted, the number corrected for the dilution, and the mean result determined.

In collecting samples from a cistern, pool, or well, from which the test-specimens are to be taken at different depths, it is customary to use glass-stoppered bottles arranged in the following manner: The bottle and stopper having been sterilized, the operator carries it closed to the place of collection, where a stout cord of sufficient length to reach the depth desired is attached to the stopper and a like one tied to the neck of the bottle; and a convenient piece of rock or other weight is attached to the bottom of the bottle. It is lowered to the desired depth, the stopper withdrawn by a pull on the cord attached to it, and the bottle allowed to be filled with the water. It is now quickly pulled to the surface, but steadily and without jerks, a little of the contents poured over the stopper to rinse off the water of the upper strata through which it was drawn, and the stopper adjusted. It is probable that a little

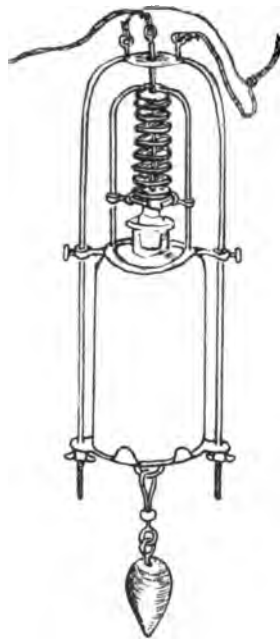


FIG. 42.—BOTTLE IN FRAME, ARRANGED FOR COLLECTING WATER FROM DEFINITE DEPTHS AND REPLACING STOPPER AFTER ENTRANCE OF WATER.

water from the overlying layers will have entered the bottle, but this amount may be disregarded, the full condition of the bottle and the small size of the neck preventing much displacement. To prevent this source of error special forms of collecting apparatus are sold in which it is possible by mechanical device to replace the stopper while the bottle is in the depths (Fig. 42). Samples are taken from the top, middle depth, and bottom, and each used in making cultures as above described, dilutions being made as above, if necessary. Either the mean of the results obtained or a statement of the result for each depth should be indicated in the report of the analysis.

It is to be remembered in all such procedures that the number of organisms arrived

at from counting the number of colonies developing in the cultures probably represents incompletely the real proportion of bacteria in the sample. Each colony is supposed to represent one original bacterium, but if the diffusion has not been entirely successful, a clump of organisms might well have developed into a single colony; moreover, it is quite probable that not all of the germs will have developed on the medium selected and at the temperature to which the cultures were exposed. Close watch should be maintained upon the preparations so that all the colonies are included in the enumeration, a magnifying glass being used to detect the smaller ones. Where greater exactness is demanded it is well that not only gelatine preparations be made, but that agar be also inoculated, plates of which, after being grown for several days in the room temperature, are placed in the incubator in order that organisms growing only in warmth may be afforded opportunity for development. By this measure one may evade the difficulty which is apt to arise from the liquefaction of the gelatine by the bacteria, a source of much confusion in many cases. As a rule the common water bacteria develop best in these cultures during the first few days while exposed to the temperature of the room, the incubator preventing their free development.

Especially upon these agar preparations at incubator temperature is there probability of appearance of colonies of such pathogenic and parasitic organisms as may have been present in the sample. In ordinary investigations they are very likely to be overlooked and lost. Of course, their recognition must depend largely upon the skill, experience, and watchfulness of the observer; but in the midst of the hundreds of colonies of much the same general appearance on the plate, even should they appear they may be unrecognized by the well-informed and skilled. In order to favor their recognition, it is best to induce their increase in the sample of water in some way before subjecting it to culture, and then make cultures on agar preparations at incubator temperature. This modification of the original sample is not to be made, of course, until after the preparation of the cultures above described, which are intended for the exhibition of the common bacteria and for their enumeration. Thereafter there may be added to the remainder of the sample in the collecting bottle either peptone (one per cent.) and sodium chloride (one-half per cent.) or a small amount of sterile bouillon; after which the bottle and the contents are placed in the incubator for twenty-four or forty-eight hours for the more satisfactory development of the suspected bacteria. Under such conditions of added nutrition and warmth the typhoid organism, the colon bacillus, and other important forms are apt to increase to a marked extent, while the common water germs develop but poorly or are prevented entirely. Diffusion or smear preparations on solid media are now made from this and grown in the incubator. The final recognition and identification of such organisms must depend thereafter upon the experience and skill of the observer, the separation of the typhoid organism from the other varieties of the colon group being a difficult and often uncertain task, although the recognition of the organisms as members of this group is not difficult and quite convincing as evidence of the fecal contamination of the sample of water from which they were obtained.

Milk Examination.—Milk as obtained directly from the cow is invariably contaminated with bacteria which have been on the surface of the teats or which have been growing in the milk close to the openings of the milk ducts. Under the most rigid cleanliness the fresh milk is apt to contain from ten to twenty thousand bacteria to the cubic centimeter; and milk is often supplied to purchasers which contains as many millions to the same volume. The New York Board of Health prescribes as a limit for good milk two hundred thousand to the cubic centimeter. For the most

part these bacteria in fresh milk are non-pathogenic cocci, although occasionally the mycobacterium of tuberculosis is met with as a serious contamination; and from air or water contamination a variety of more or less serious infections may be met with in older samples. The analysis of milk by culture follows the methods used for water examination. The milk is always diluted and very small quantities of the dilute sample (1:100 or 1:1000) employed for diffusion in the nutrient medium, both for the purpose of reducing the number of the bacteria and the confusion of their colonies, and to prevent an annoying turbidity in the medium caused by the fat of the milk.

Soil Examination.—The surface of the ground for a variable depth, depending on its porosity and the amount of surface contamination, is occupied by a great variety of organisms, growing upon the organic matter diffused by drainage through the pores of the ground. These organisms ordinarily are found in profusion in the upper three or four feet, becoming less numerous at greater depths; and after the intervention of a bed of rock or firm clay they are usually no longer met with. For the most part they are saprophytes of little medical interest, concerned in the conversion of the organic substances in the ground into carbon dioxide, water, and ammonium; but the organisms of tetanus, malignant edema, black-leg, and several other affections are also often encountered, and from recent contamination with effluvia from diseased



FIG. 43.—HARPOON FOR COLLECTING SOIL FROM BELOW THE SURFACE OF THE GROUND.

individuals the germs of typhoid fever, anthrax, cholera, and a number of other diseases may also temporarily exist. One especially important group of soil organisms are the *nitrifying bacteria*, universally present, but not capable of cultivation upon the ordinary media, and hence not recognized in the usual bacteriologic analysis of soil. They are concerned in the oxidation of ammoniacal compounds in the soil into nitrous and nitric acid and their salts, which are then utilized by the growing vegetation; hence their importance to the agriculturist from their rôle in the fertilization of the soil. They are grown upon solutions of ammonium sulphate and phosphate of potassium, or upon a mixture of these together with magnesium sulphate, calcium chloridé, and sodium carbonate added to silicic acid, this mixture producing a mass of gelatinous consistence.

In examining the soil, specimens are taken from different depths; a convenient harpoon for obtaining the samples is that shown in figure 43. A definite amount of the soil obtained is diffused in a known quantity of sterile water, and a measured quantity of this dilution with the bacteria scattered through it is planted by diffusion in the nutrient medium. This is then plated in one or other of the usual manners and set aside for growth; from the number of resultant colonies the number of bacteria in the original specimen is calculated; and the further study of their characteristics pursued.

Material Obtained from Diseased Individuals.—In the bacteriologic study

of the various infectious diseases the first object of the investigator is *the discovery, in the tissues or fluids of the diseased individual, of a probable microorganismal agent*. For the accomplishment of this, the first of the familiar postulates of Koch, two methods of procedure are available and both should be pursued: the examination of the diseased material by the microscope and the practice of inoculation of nutrient media with such material. The examination by the microscope may be directed to the fresh, unstained matter or, better, to stained films smeared and fixed upon a slide or cover-glass (see Smear Preparations, Lesson VII).

For successful culture of the pathogenic bacteria blood-serum probably offers the greatest promise of any of the common nutrient media; but, as in any other study concerned with unknown forms of micro-organisms, one should at least in the preliminary inoculations have recourse to the entire range of laboratory nutrients, several inoculations being made upon each type to insure the fullest chance of avoiding failure. Here may be recalled the method of McLaughlin, originally devised and employed by him in the study of dengue fever, of employing as the artificial nutrient the natural body material supposed to be the habitat of the organisms sought. This investigator withdrew from veins into sterile glass tubes some of the blood of the infected subject and with it the organisms he has described, thereafter keeping the blood in the collection tube in the incubator for culture of the parasites. The method has wide application, especially for infected blood and fluid exudates, excretions and secretions, but also for infected solids, and has been utilized by a number of workers in a variety of ways. Whatever media be employed, the various methods of inoculation possible for each should be practised and the inoculated preparations subjected, as hereafter suggested, to body temperature in the incubator as well as to room temperature, and to anaerobic conditions as well as to the ordinary atmosphere.

In case of exposed lesions, as the surface of an ulcer, or a diphtheritic throat, either in the living or the dead subject, the wire loop or the swab may be well used for taking up the infected matter for inoculation. In obtaining blood, it may be removed from one of the large superficial veins of the arm of the living subject by means of a sterile hypodermic syringe or from the pulp of the spleen, after thorough disinfection of the skin and, in very cautious operations, after a small superficial opening has been made through the sterilized skin by a sterile knife. Other fluids, as spinal fluid, peritoneal or pleural exudates, or the contents of an abscess, may be obtained in the same way. When practicable for collection, Sternberg's bulbs, capillary tubes, or pipettes may offer some advantages over syringes for subsequent convection, inoculation, or culture. From the material thus procured smears and stabs are to be made in solid media and diffusions in liquefied agar and gelatine, the latter being subsequently plated. When solid diseased tissues are removed by the surgeon and are to be submitted to bacteriologic examination, they should at once be placed in sterile jars and hermetically sealed for convection to the laboratory. After reception in the laboratory, as soon as possible a knife-blade should be heated to red heat and plunged into the mass, thus producing a sterile track into the midst of the tissue where there cannot have taken place any air contamination after removal from the body. Along this track a heavy platinum needle, heated to a red heat, is carried, plunged through the eschar caused by the knife, and allowed to cool in the interior. When cool, it is pushed beyond the zone influenced by its own heat and twisted about in the structures until it has in all probability become infected. It is then withdrawn and used in making the usual smear, stab, and diffusion inoculations.

At autopsies it should be made a rule to obtain the material for inoculation as

soon as possible after death in order to prevent the appearance of the organisms of putrefaction as a source of error and confusion; and wherever an early operation is impracticable the cadaver should be kept on ice or in a refrigerated room to avoid the same trouble. Inoculations should be made from all special points of involvement, and from the blood of the heart, spleen, any exudates in the tissues or body-cavities, the liver and the kidneys as a matter of routine. In obtaining the material from such sources the same precautions to prevent contamination should be observed as directed above when dealing with tissues removed surgically, penetration into the interior of cavities and through the capsules of organs being accomplished by a hot blade, and the material removed by sterile syringe, needle, or other appliance. Instead of an ordinary platinum needle the heavy platinum harpoon of Nuttall, in a metal or glass handle, is of advantage in that it is not easily bent in penetration into firm tissue.

After inoculations have been made from the various sources desired, not before, films should be smeared upon glass covers or slides from each examined part for microscopic study.

CULTIVATION OF INOCULATED MEDIA.

The conditions for growth and multiplication of bacteria are the availability of a proper *nutrient* and *moisture*, and the presence of a suitable *temperature* and *atmosphere*. In addition, *quiet* and *absence of extremes of light or darkness* should be included. These conditions are sought to be obtained in the measures taken for the successful cultivation of the germs in the laboratory.

1. **Nutrient.**—Sufficient has been said concerning this feature in the consideration of the various media to indicate that the important elements of nutrition—carbon, hydrogen, oxygen, and nitrogen, as well as traces of other elements (as iron, sulphur, phosphorus, etc.)—are obtainable from the media themselves. There are numerous organisms which grow upon much more simple preparations than the ones described, as upon solutions of salts; and there are probably a number for whose artificial cultivation the media thus far devised have not approached sufficiently the material afforded by the living body. The last are truly obligate parasites. The intricate changes which are accomplished by the organisms in their appropriation of nutrition and growth in these laboratory cultures are, of course, but poorly understood. They are mainly reduction processes, oxidations, and hydrations.

2. **Moisture.**—The moisture present in a medium is an essential element; experiments in which it has been purposely diminished show that its loss exerts a disturbing influence upon the development of the organisms, growth being most vigorous when seventy or more per cent. of water is present in the composition of the medium, and practically disappearing when it has been reduced as low as forty per cent. Upon this depends the success of practical preservation of foods and other organic substances by drying. The drying of the medium does not necessarily destroy the vital possibilities of the bacteria in the substance, even though there be no spores to account for the persistence; and after months of drying it is often possible to see profuse growth appearing after the addition of water or bouillon to a dried culture. In order to prevent the loss of moisture from the culture tubes, particularly those in the incubator, the warmth of which would otherwise much hasten desiccation, a number of devices are occasionally resorted to. The tubes may be capped with rubber covers or closed with rubber stoppers (as for preservation of the uninoculated nutrient media); or the atmosphere of the incubator (or of whatever other inclosure in which the cultures

may be kept) may be kept moist by placing a small open dish of water in the chamber with the cultures, thus lowering the rate of evaporation from the inclosed media. In potato dishes the purpose of the layer of moistened filter paper upon the bottom of the dish is to prevent any rapid desiccation of the potato substance. The addition of glycerine to agar or serum, aside from its special nutritive value, affords an influence toward the retention of the moisture of the medium.

3. Temperature.—The influence of temperature on the growth of bacteria is an important one, leading to the recognition of three classes of these organisms, according as the temperature most favorable for their development is low (room temperature, 15°–20° C.), about body temperature (35°–38° C.), or high (50°–55° C., or above), known respectively as *psychrophilic*, *mesophilic*, and *thermophilic* bacteria. To the first belong many of the saprophytes, as most of the common water bacteria; to the second, most of the bacteria parasitic and pathogenic to animal life; to the third, the putrefactive bacteria, many of the bacteria found in the ground, and others. In relation to any one bacterium it is customary to speak of that lowest temperature at which it first and feebly grows as its *minimum temperature*; that at which it best develops, as the *optimum temperature*; and that highest temperature at which it continues, although feebly, as its *maximum temperature*. The entire range from minimum to maximum for most bacteria will be found to extend about thirty degrees, a few having much wider range, and a number, particularly pathogenic varieties, a more restricted one. Some forms are capable of development in ice or snow, others at the highest heat of sun exposure; whence it will be easily appreciated that the general range for the whole group must extend from zero to as high as 60° or 70° C. For most forms the optimum ranges from room temperature to body temperature.

In their artificial cultivation the temperature sought to be provided is the optimum, or that most favoring their growth, particularly for those easily disturbed by variations from this, as is the case with many of the pathogens. While slight differences may not be sufficient to destroy the vitality of these forms, their effect (especially if increased) is to interfere with the rate of development and often to occasion important alterations in the general functional activities of the germs. Sporulation may take the place of the ordinary vegetative multiplication, variations in the shape and size of the individual organisms may be induced, changes in the degree of virulence of the pathogenic varieties may occur, as well as other modifications of their normal manifestations. These changes are included under the term *pleomorphism* of bacteria, which may be induced by important changes from any of the normal conditions of life as well as by alteration of the culture temperature. Such pleomorphism does not involve in any instance a change of specific type, cessation of growth and of life occurring rather than absolute loss of type. Such variations may, however, persist through generations under like modifications of life conditions. The anthrax organism, for example, is thus lowered in its virulence by growth at a temperature of 40° C. (optimum, 37° C.) in the manufacture of the vaccine commonly used in the immunization of flocks and herds against the virulent disease.

Usually two temperatures are available in the bacteriologic laboratory, that of the room (15°–20° C.), and the body temperature (37° C.), which is maintained by means of an incubator and is commonly spoken of as *incubator temperature*. By means of incubators any desired temperature may be obtained and maintained uniformly; and for special purposes in the larger laboratories there are usually provided incubators in which temperatures are maintained at ranges below (30° C.) and above (40°–45° C.) that of the generally used warm chamber (37° C.). An incubator is essen-

tially an inclosure the temperature of which is maintained at a uniform degree by artifice of some sort. The form most efficient and usually used in the laboratory is a chamber surrounded, except on the side of the door, by a water-bath (Fig. 44), the source of heat being a small, constant flame adjusted beneath the apparatus. For better preservation of uniformity there is usually an incasement of asbestos felt on the exterior, except over the bottom where the flame is applied. A double door, the inner portion of glass (for inspection of the interior of the chamber without exposure) and the outer of metal

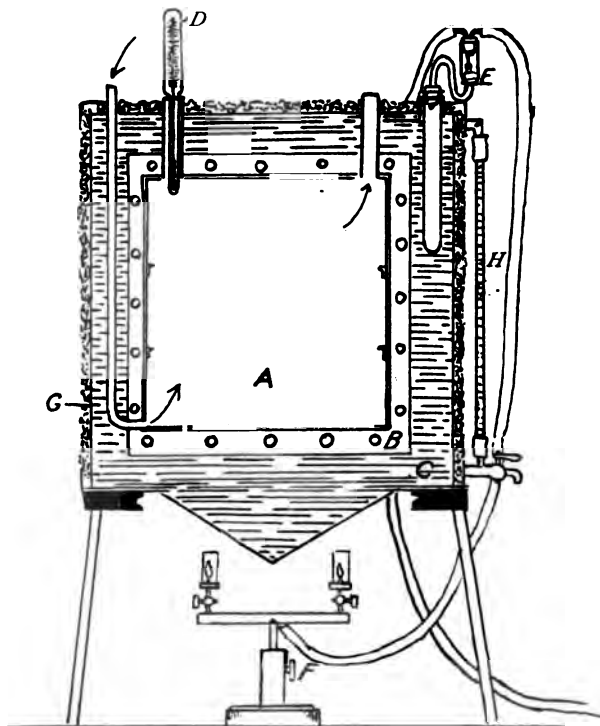


FIG. 44.—SECTIONAL VIEW OF INCUBATOR.

A. Incubator chamber. *B.* Ventilation wall. *C.* Water-bath. *D.* Thermometer. *E.* Thermostat. *F.* Microchemical burners. *G.* Felt covering of incubator. *H.* Water gauge.

and felt, allows access to the interior; and a small opening is provided through the top for accommodation of the thermometer, which extends into the interior. Several openings at the top lead into the interior for ventilation, and into the water-bath for filling, cleaning, and the introduction into the warm water of a *temperature regulator* (*thermostat*). This last device is an appliance for the automatic regulation of the size of the flame beneath the incubator, causing it to decrease as the warmth of the water increases, or to increase as the temperature of the water falls. There are a number of forms applicable to gas or to lamp flames, as well as special devices for the regu-

lation of the heat obtained from electricity or from steam or hot-water pipes; the general principle of all depending upon the expansion or contraction of volume of some solid or liquid upon the application of heat or loss of surrounding temperature. A gas thermostat, as the cheap and efficient form shown in figure 45, may be described as illustrative. The tube *A*, a large test-tube, is connected by perforated rubber stoppers, and the ω -shaped tube, *B*, with the glass cylinder, *C*, the arm of *B* projecting some distance into *C*. This cylinder, *C*, is closed above by a rubber stopper having double perforation.

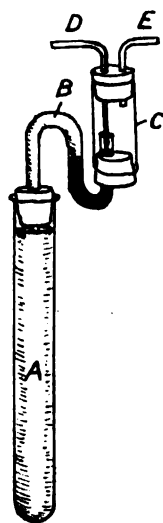


FIG. 45.—THERMOSTAT.

- A. Tube containing expansile liquid.
- B. ω -shaped tube containing in shaded part mercury.
- C. Cylinder serving as gas chamber.
- D. Feed tube.
- E. Escape tube.

One of the tubes, the feed tube, extending through this stopper reaches into the arm of the tube *B*, projecting through the stopper in the bottom of *C*. Its lower end is ground to a bevel, and there is a tiny hole bored through its wall a little distance above the beveled end. The other, the exhaust tube, extends but a short distance into the cylinder. The test-tube, *A*, is filled with a mixture of alcohol and ether or any other fluid having a large coefficient of expansion by heat, the mixture extending well into the ω -tube. The dependent curve of the latter tube contains mercury, whose level joins the fluid in the other arm and serves to inclose it and prevent its evaporation. The beveled end of the supply tube reaches to the free surface of the mercury in the ω -tube. The large tube (*A*) containing the expansile fluid is now inserted into the water-bath of the incubator, the rest of the apparatus projecting into the outer atmosphere. The supply tube is connected by means of a rubber tube with the gas-pipe of the laboratory, and a tube leads from the exhaust tube to the burner beneath the incubator. As the water of the bath rises in temperature its warmth causes the fluid in *A* to expand; this in turn forces the level of the mercury in the ω -tube to rise higher and higher about the beveled end of the supply tube, thus narrowing more and more its orifice over the mercury. To offset entire closure of the opening over the mercury, otherwise completely cutting off the gas-supply and extinguishing the flame beneath the incubator, the tiny hole in this tube has been provided. As the orifice of the supply tube becomes less and less by the elevation of the mercury column, the gas passing to the flame is diminished and the flame lowered. Gradually the temperature of the water in the bath decreases and with this the fluid in *A* diminishes in volume and allows the level of the mercury to fall away from the beveled end of the supply. Again a greater volume of gas passes to the burner, the flame rises, and the temperature of the water in the bath increases. By a careful adjustment, if the pressure of the gas be even, the temperature of the bath, and consequently of the incubator chamber, may be maintained indefinitely within a variation of less than one degree. For delicacy of the apparatus the volume of the expansile fluid in *A* should be large in proportion to the volume of mercury which it is made to lift in the ω -tube.

One of the most annoying interferences with the maintenance of an even temperature arises from inequalities in the pressure of the gas in the general supply pipes. To obviate this the pipe connected with the incubator should be connected as directly as possible with the main (near the meter) and not attached to a series having many

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side connections. The variations in pressure in the mains leading directly from the gas works are usually gradual and a good thermostat is capable of close regulation from such a source without the need for further intervention; but to prevent the sudden changes in a house pipe carrying a large number of burners in frequent and intermittent use, a *gas-pressure regulator* should be interposed between the thermostat and the pipe from which the incubator is supplied. A number of devices are used for this purpose, but the adaptation of the principle of an ordinary gas tank having a constant weight superimposed, as in the Murrell regulator (Fig. 46), is probably the most efficient form.

The cost of the bacteriologic incubators, as well as of other set apparatus used in well-equipped laboratories, is often prohibitive to private individuals, who in the course of their medical practice might otherwise be anxious and able to conduct investigations in clinical bacteriology to the advantage of themselves and their patients, and to that of science in general. In this connection it may therefore be suggested that the ordinary egg incubator, with a lamp as the source of heat and the thermostat

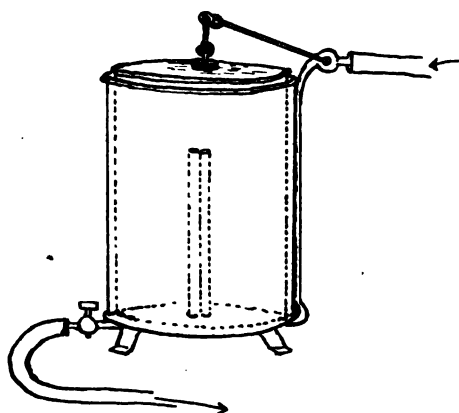


FIG. 46.—MURRELL GAS PRESSURE REGULATOR.

used in such an appliance, may easily be arranged to answer every probable demand. There is shown in the accompanying diagram the plan of a cheap incubator of large size used by the writer for class work, which can be duplicated for a comparatively small sum (Fig. 47). The arrangement of the hot-water tank on the inside of the incubator chamber, instead of as an outside jacket, is not so efficient as the latter in the maintenance of an even temperature through the whole inclosure, but it is a much cheaper mode of application. It has been possible to keep the temperature of selected parts of the apparatus uniform within two degrees, and if a proper circulation of the heated water were arranged by a series of tubes this variation could be reduced. The degree of warmth for each part of the chamber is, however, constant, the warmest part being in the lower tier of wire cages near the tank, and the coolest near the top and front. Every incubator should occupy a place in which as few atmospheric draughts as possible prevail, to escape disturbances of the flame and to lessen the loss of heat by convection. It is well to have a small room, separate from the general laboratory, devoted to the accommodation of the incubators and arranged so as to best protect them.

As an extemporization in clinical work it may be possible to utilize some nook close to a range or house furnace for the accommodation of culture tubes; but this is at best precarious. One or two tubes of infected medium in a small metal box, wrapped in several layers of cloth or paper, and put in an inner pocket, carried to bed at night, may be successfully incubated by body warmth should necessity place one in extremes.

4. Atmosphere.—From their relations to the oxygen of air bacteria are usually classified in three groups—*obligate anaerobic bacteria* (*obligate anaerobes*), *obligate*

aerobic bacteria (obligate aerobes), and *facultative anaerobic bacteria (facultative anaerobes)*. The first group are unable to vegetate or sporulate except in the absence of free oxygen; the second, except in the presence of free oxygen; the third group, while usually developing in the presence of the atmospheric oxygen, are able to maintain their activities in its absence. Probably this division should not be accepted in an absolute sense, since in case of many of the known aerobes there is the power of adaptation to atmospheres containing less and less oxygen, and the same is true of many anaerobes grown in atmospheres of nitrogen or hydrogen to which are gradually added small proportions of oxygen. It has been noted, too, that when living in a culture with aerobic bacteria it is sometimes possible that development of associated anaerobes will occur in the ordinary atmosphere, as seen clinically in the association of the bacillus of tetanus with the pyogenic cocci in superficial wounds. So, too, it has been noted that the

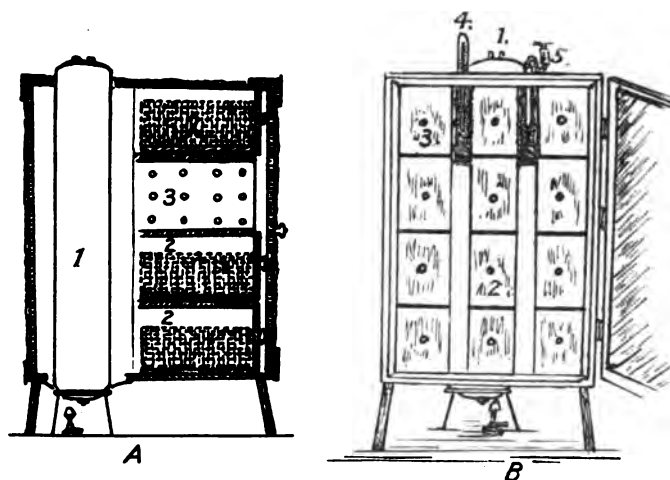


FIG. 47. LARGE CLASS INCUBATOR WITH INTERIOR WARM-WATER TANK, AND WIRE-CAGE DRAWERS, WALL OF WOOD, COVERED WITH FELT.

A.—Side section. 1. Water-bath. 2. Wire-cage drawers. 3. Partition between rows of drawers, perforated for proper circulation of warm air.

B.—Front view, open door. 1. Water-bath. 2 and 3. Wire-cage drawers; front of partitions removed above to show insertion of (4) thermometer and (5) thermostat.

addition of certain substances, as sodium sulphide (one or two drops of a ten per cent. solution to ten cubic centimeters), to ordinary bouillon or gelatine will enable many anaerobes to develop in such media in the ordinary atmosphere. On the other hand, an excessive proportion of oxygen in the atmospheres of cultures of aerobes may modify their vital phenomena (as in the reduction of the virulence of *Bacillus anthracis* by excess of oxygen) or eventually destroy them.

Among the anaerobic organisms are many of the soil bacteria, that of lockjaw being of much pathologic importance; among the aerobes are most of the common saprophytes, while in the group of the facultative anaerobes bacteria pathogenic and parasitic to animal life are found.

Atmospheres of nitrogen or hydrogen are well suited for the growth of anaerobic

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bacteria, and it is said that hydrogen sulphide is likewise favorable; carbon dioxide is restrictive. Mere reduction of atmospheric pressure by the air-pump may be employed for the purpose, but an actual vacuum is not conducive to their development. It is probable that, like other living things, these anaerobes actually require oxygen for their energies, but are able to obtain the small amounts needed from the material upon which they are grown.

Various devices and special measures have been suggested for the development of anaerobic organisms. In many of these, jars of some form or other are prepared so that an atmosphere of nitrogen or hydrogen may be introduced to replace the ordinary air. A simple type of such jar is shown in Fig. 48. The jar, cover, and rubber ring should first be disinfected and rinsed in boiled water. The inoculated tube cultures (without cap or rubber stopper), small plate cultures, or open Petri dishes of small size, are placed in the jar, the cover adjusted (taking care that the long supply tube extending from the cover does not come in contact with plate or dish media) and fastened hermetically. Hydrogen gas is generated in a Kip generator (Fig. 49) or other apparatus from dilute sulphuric acid and zinc clippings (gas preferably washed by being passed through one or two Woulff bottles containing water), and passed

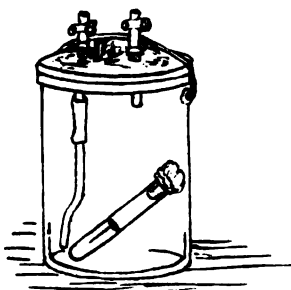


FIG. 48.—ANAEROBIC JAR.

into the supply tube, the clamps of both the supply and the escape tubes being loose. The hydrogen entering the lower part of the jar gradually displaces the air which passes out through the exit tube. It is possible to determine the purity of the hydrogen gas after it has passed through the jar (air expelled) by collecting some of the escaping gas under water in a test-tube and applying a flame. If no explosion follows, the air has all been expelled. However, if one will allow the gas to flow freely for about five minutes, then close the clamps for a time, and after five or ten minutes more (allowed for diffusion of gas in the tubes of the cultures) repeat the operation, he may be confident of having obtained the desired condition. The Hoffmann clamps are then tight-

ened, and the jar placed in the proper temperature for the best development of the organisms.

Another simple method is to rely on the removal of the oxygen from the atmosphere by means of an alkalized solution of pyrogalllic acid. Buchner suggests that a large test-tube be employed, the inoculated culture tube being placed in the interior. A rubber stopper is obtained by which to seal the large tube. Several cubic centimeters of a saturated solution of pyrogalllic acid are introduced into the large tube outside the culture tube, and alkalized by adding an equal amount of a strong (forty per cent.) solution of sodium hydroxide, and the rubber stopper at once applied. The oxygen is absorbed by the mixture, leaving an atmosphere mainly composed of nitrogen.

The writer has for a number of years employed with some success the measure for excluding the atmosphere from the tube cultures by filling the tube after a stab or diffusion inoculation has been made with the same medium as that in the tube or with paraffine still liquid, but near the temperature for solidification. Sterile oil may be poured over the inoculated medium for the same purpose. On plates a sheet of sterile isinglass or thin glass closely applied to the surface of the medium may sufficiently exclude the air from the underlying medium to be followed by growth of anae-

robic varieties. Salomonsen's capillary tubes drawn full of the inoculated medium, and the ends sealed, will often show marked growths of the anaerobes; and if in the fermentation tube growth be limited to the closed arm of the apparatus, it may be inferred that the organism present is capable of anaerobic development.

5. **Light.**—Direct sunlight is destructive to most organisms, even diffuse light if moderately strong being restrictive to many. On the other hand, absolute and constant darkness is not favorable for their best development, the most suitable con-

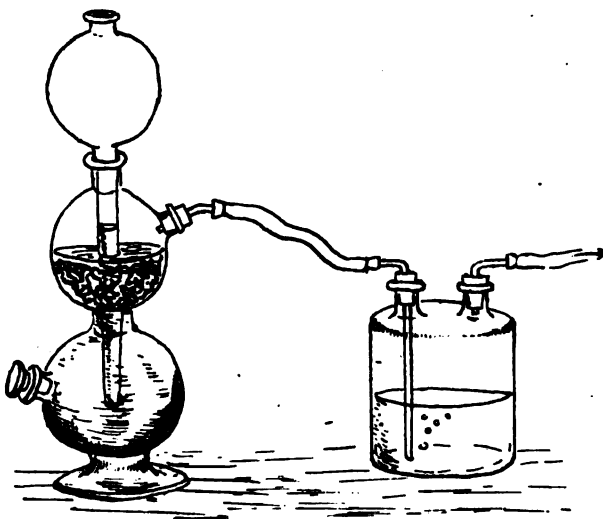


FIG. 49.—KIP GAS GENERATOR AND WASH-BOTTLE.

dition being an extremely weak, diffused light alternating with periods of darkness. Cultures in the incubator are thus properly placed; and it should be a rule that cultures growing at room temperature be placed in a rather dark nook for their best growth.

THE NUMERICAL ESTIMATION OF BACTERIA.

Sufficient reference has been made in connection with the above instructions as to the mode of analysis of air, water, milk, and soil to have the student understand that for this purpose diffusion inoculations of known small quantities of the original infected material or of known dilutions of such matter in sterile diluents are first to be made. As suggested, it is best to make a series of such dilutions, diffusing each in the medium so as to have a series of cultures for comparison of results. After diffusion in the medium (usually liquefied gelatine) in a culture tube, the mixed contents are to be plated, either in plates, in Petri dishes, or in the interior of the tube (Esmarch tube), the inoculated bacteria being thus disseminated over the surface upon which the film of medium is distributed. It is to be presumed that upon placing these plated cultures in proper temperature, atmosphere, and in a dark place, each bacterium will grow into a colony more or less separated from its fellows. The time permitted for growth must vary somewhat with circumstances, but forty-eight or

seventy-two hours are usually sufficient for the appearance of the colonies so that they may be detected. Should gelatine have been selected as the medium of growth, the confusion arising from liquefaction of the medium may require earlier counting, or that recourse to agar preparations as media be had.

Should the number of colonies present in the dish, plate, or tube be small, there can, of course, be no difficulty in their enumeration; but one must always be careful not to overlook small colonies, and a magnifying glass should be used for their detection, and in order not to mistake bubbles or small dirt particles for colonies. If the preparation be placed over a dark surface, as black paper or cloth, the colonies stand out more clearly and are more easily recognized.

If, however, there be great numbers of bacteria present, recourse must be had to some device to aid the worker. Thus, a piece of black paper may be ruled with white lines into squares having each side one centimeter in length, and this then placed beneath a plate or dish culture, through which the lines are visible, dividing the culture into square centimeters. One may, if time and inclination agree, proceed to determine the number of colonies in each of these squares in regular system until

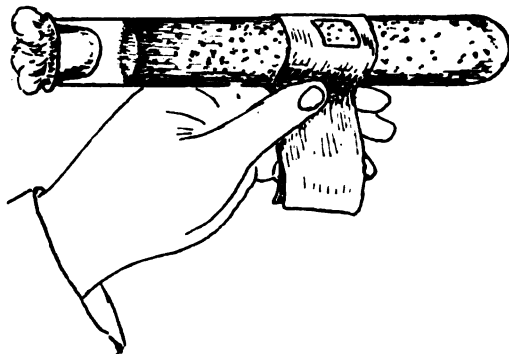


FIG. 50.—MODE OF COUNTING COLONIES IN AN ESMARCH TUBE.

all have been included in the count. This is the safest procedure if there be not too many colonies. Or, having counted the colonies in a number of squares in different parts of the culture, a mean may be struck for one square centimeter. The square surface of the culture being known, the calculation for the whole culture is easily made. For counting the number of colonies in a Petri dish a black surface, marked off by concentric circles, radial lines, and subdivisions, into divisions each one square centimeter in area, may be found more convenient than the squarely ruled surface. In counting the colonies in a rolled tube a strip of paper, in which an opening of one square centimeter size has been cut, may be folded about the tube (Fig. 50); the colonies showing in the opening counted, and the slip removed from place to place until ten or twelve separate square centimeters have been gone over. From the inner diameter of the tube may be calculated the circumferential measure of the film (diameter multiplied by 3.14159); this is multiplied by the extent of the film in the long axis of the tube (exclusive of the curve of the bottom, which may be neglected to make correction for the error arising from the fact that the inner circumference of the film is less than that of the tube) and thus the square surface of the film obtained. The number of

colonies calculated for one square centimeter, multiplied by the number of square centimeters in the film, furnishes the total number of bacteria in the material inoculated. Or, if one wishes, the outer surface of the tube may be laid off in squares by longitudinal and circumferential lines, a wax pencil being used to write on the glass, these serving as the squares laid beneath an ordinary plate or dish culture. After a proper count of the colonies in each culture of the several dilution inoculations has been made, and this number multiplied by the coefficient of dilution of the original substance, the mean of the results should be taken as most nearly representing the correct number sought. However, it should be recalled that the figures obtained are really only approximations, and are understatements rather than exaggerations, owing to the failure of this or that bacterium to develop into a recognizable colony for some reason or other, and owing to the fact that some colonies may represent, not isolated organisms, but clumps of germs.

Exercise 30.—Under the eyes of the instructor let each student in turn make an inoculation of tubes of solid medium by stroke (using plain needle), smear (using loop), and stab inoculations; and of liquid medium by diffusion inoculation (using loop). In this it should be seen that the student properly holds the tubes from which material is obtained and to which inoculation is to be made; that he removes the stoppers correctly and completely, and disposes of them properly; that he flames the needle properly both before and after inoculation, flames the mouth of tube and stopper before readjusting the latter, and that the whole process is carried out without undue exposure to contaminations.

Exercise 31.—Each student prepare a swab, sterilizing it for use in obtaining material from the throat of a diphtheritic patient.

Exercise 32.—Prepare from soft glass tubing three capillary pipettes, the capillary portion of each of which shall be uniform for at least twelve inches; graduate each into tenths of a cubic centimeter, properly preparing it for sterilization in the oven (rinsing in alcohol and ether, and plugging end with cotton); thereafter sterilize in oven for future exercise.

Exercise 33.—Each student prepare one Sternberg bulb, at least one-half inch in diameter of bulb and having a capillary tube of at least two inches in length.

Exercise 34.—By means of sterile pipette measure one cubic centimeter of milk and add it to nine cubic centimeters of sterile distilled water in a small sterilized flask, mixing by gentle agitation. From the mixture, by means of a sterilized capillary pipette, remove 0.1 cubic centimeter and transfer to a tube of liquefied gelatine (kept liquid in warm water at 30° C.). At once diffuse, and, following instructions given above, distribute the diffusion upon a sterile glass plate and place it in a culture dish for development. Remove from the remainder of the decimal dilution, by means of the sterile pipette, one cubic centimeter of the dilute milk; with assistance of a fellow

student at once rinse out the flask with sterile distilled water, placing thereafter in it nine cubic centimeters of sterile distilled water. To this add the one cubic centimeter of the decimal dilution, thus preparing a one per cent. dilution, diffusing thoroughly by agitation. From this second dilution remove with sterilized capillary pipette 0.1 cubic centimeter and transfer to a tube of liquefied agar (kept liquid in water-bath at 45° C.); diffuse by agitation, and transfer the contents to a Petri dish, in which it should be quickly cooled so as to harm as little as possible the bacteria by the temperature of the agar, placing the dish after solidification in the incubator. From the one per cent. dilution remove with a sterile pipette one cubic centimeter of the dilute milk; again quickly rinse out the flask with sterile distilled water, and place nine cubic centimeters of the sterile distilled water in the flask. Add the one per cent. dilution to the latter, thereby making a one in a thousand dilution. Of this, with sterile capillary pipette inoculate 0.1 cubic centimeter to a second tube of liquefied gelatine and roll over ice to make an Esmarch tube. Put aside in a dark place at room temperature for further development. Observe each culture at close of the first, second, and third days, and at the close of the last period count the number of bacteria obtained in each dilution. Calculate therefrom the number in one cubic centimeter of the undiluted milk. (Should liquefaction of medium endanger the definition of the colonies before the seventy-second hour shall have been passed, count at an earlier period as conditions require.)

Note the differences shown by the colonies on the agar in the incubator and those of the gelatine plate and tube at room temperature.

(As far as possible let the instructor witness the various steps taken by each student in the processes of dilution, inoculation, and the different modes of plating the inoculated media, to insure all necessary precautions against contamination and to correct errors of manipulation.)

Exercise 35.—Make an inoculation of one cubic centimeter of water from the tap or from a cistern into a tube of liquefied agar; diffuse by agitation; transfer to a Petri dish; grow in incubator. Make a similar inoculation in a tube of liquefied gelatine; transfer to Petri dish; place in a dark place at room temperature for development. Compare the rate of development of these psychrophilic bacteria at the two temperatures. Are those on the agar different in gross appearance of colonies from those on the gelatine?

Exercise 36.—Inoculate two tubes of solid blood-serum by smears from a known culture of *Mycobacterium diphtheriæ*. Let one remain at room temperature; grow the second in the incubator at body temperature.

Note difference in the cultures at close of first, second, and third days. To what group is the organism to be referred from its optimum temperature?

Exercise 37.—From a known culture of *Bacillus tetani* a stab culture in agar is made (glucose agar if preferred). It is placed for forty-eight hours in the incubator. Does development occur?

It is now transferred to anaerobic jar in an atmosphere of hydrogen and again placed in the incubator, and observed for the usual time. To what class of bacteria does this germ belong from its relations to free air access?

Exercise 38.—Two tubes of agar are inoculated by stroke from a known culture of *Bacillus typhosus*. One is placed in the anaerobic jar in an atmosphere of hydrogen, the other retained in the air. Both are placed in the incubator. Note the results of growth at the close of the first, second, and third days. To what group does this organism belong from its relation to air?

Exercise 39.—From a known culture of *Pseudomonas pyocyanea* (*Bacillus pyocyaneus*) make a stroke inoculation on a tube of agar. Place in an atmosphere of hydrogen for two days at incubator temperature. Does growth occur? Transfer the tube to the open air in the incubator. Observe the results after one, two, and three days. Is this organism an obligate anaerobe, facultative anaerobe, or obligate aerobe?

Exercise 40.—One or two loopfuls of fresh pus are diffused in a few cubic centimeters of sterile water. Smear preparations are made on two agar "slants" (tubes in which the medium has been solidified in slanting position). One is placed in the sunlight near the window, the other in a dark place at the temperature of the room. Compare the two tubes each day for three days and note results. Can the culture exposed to sunlight be developed by transfer to darkness and warmth, or are its organisms dead?

LESSON VI.

STUDY OF GROSS APPEARANCES OF BACTERIAL CULTURES.

Upon development of bacteria inoculated upon a nutrient medium there appear as a result of multiplication of the individual organisms more or less isolated colonies. A *colony* is a focus of growth, or group of bacteria, recognizable by the eye, unaided, or aided by low powers of the microscope. Should such a colony, as is usually the case, be composed of bacteria of the same type, arising from the multiplication of a single original germ, it may be spoken of as a *pure colony*; while if there be confused in the group several types, resulting from the merging together of minute foci of development of closely situated but different germs, it may be termed an *impure* or *mixed colony*. In liquid media the chances for diffusion of the organisms by agitation, extension of growth, or motility of the germs are so marked that isolation of the colonies and preservation of their separate characters are exceptional; while upon solid media each separate focus of growth is apt to maintain its isolation and peculiarities, whence important data for the identification of the type may be obtained. The study of isolated colonies is naturally most readily pursued in diffusion cultures on solid media, either in the films of plates, dishes, or rolled tubes, or throughout the mass of the medium, or in dilute smear cultures where the original germs have been well scattered over the surface. The massive growths of stroke and stab inoculations on solids and the unrestrained diffuse growths in liquid media present less characteristic appearances, but may in individual cases contribute important information for the same end.

An organism does not preserve an identity of appearance of its colonies and massive growths upon the various media, or when influenced by modifications of its other life conditions; and the variations produced by development upon different media and in different atmospheres and at different temperatures are likewise of importance in identification of similar but unlike types from each other. With a view of establishing these points of peculiarity it is customary, therefore, to study the appearances of growth of a given bacterium upon various media (potato, gelatine, agar, serum, bouillon, and milk), in each instance noting the characteristics shown by the different forms of inoculation, as well as the influences exerted by variations of temperature and of atmosphere upon the development in each. For complete study of all its peculiarities the organism should have been grown in agar by smear, stroke, stab, and diffusion (plate and in the mass in a tube); similarly in gelatine; by smear and stroke inoculation on potato and blood-serum; and by diffusion in bouillon and milk. Preparations of each of these should be submitted to at least two temperatures—that of the room and that of the body; and, similarly, examples of each should be placed in anaerobic conditions as well as in the ordinary air.

In every investigation the *rate of growth* (time of appearance of visible growth), *optimum temperature*, *preference of nutrient medium* and *of atmosphere* should be first noticed and recorded. Thereafter the appearances of the cultures in each medium are

in turn noted, both for the isolated colonies and the diffuse growths, including in each instance the *position of growth* (whether upon the surface or beneath the surface of the medium), the *differences apparent in surface and deep growths*, the *profuseness or poverty of growth*, the *color, shape, size, appearance of the margin, consistence, optical characteristics*, and *internal structure* of the focus of growth. The *elevation or depression of the surface growths*, *liquefaction, change of reaction*, and *other alterations of the nutrient medium*, the appearance of *gas bubbles* in the medium and any *peculiar odor* are recorded. In liquid media are noted any changes in *transparency and color*, the appearance of *pellicle or sediment*; and any *changes in the general appearances of the medium* (as coagulation of milk) are to be further observed. Modifications resulting from *age of culture* are likewise to be included in the study.

From the fact that no definite nomenclature has been adopted in this, as in other branches of physical science, for the description of cultural peculiarities, and in the absence of a definite system of observation, considerable difficulty of recognition has arisen; and it is often almost impossible, from the writings of observers working with the same organism, to be sure of the identity of their material. The personal equation in the description of the form, color, and other characters of a colony, the failure to indicate as the basis of description the exact conditions of growth, and the loose use of descriptive terms are largely the cause of this confusion; and every effort to fix a system of observation and to establish a well-defined nomenclature must be commendable. With this purpose in view the following list of descriptive terms, by permission of the author, is included in these pages, adopted from Chester's *Manual of Determinative Bacteriology*.

(For discussion of color, gas formation, liquefaction of medium, reaction changes, etc., see next chapter.)

CHARACTERS OF BACTERIAL CULTURES.

I. Gelatine Stab Cultures:

(A) Non-liquefying:

Line of Puncture:

Filiform, uniform growth, without special characters (Fig. 51, 1 B).

Nodose, consisting of closely aggregated colonies.

Beaded, consisting of loosely placed or disjointed colonies (Fig. 51, 2 B).

Papillate, beset with papillate extensions.

Echinate, beset with acicular extensions (Fig. 51, 3 B).

Villous, beset with short, undivided, hair-like extensions (Fig. 51, 5 B).

Plumose, a delicate feathery growth.

Arborescent, branched or tree-like, beset with branched, hair-like extensions (Fig. 51, 4 B).

(B) Liquefying:

Crateriform, a saucer-shaped liquefaction of the gelatine (Fig. 52, 1).

Saccate, shape of an elongated sack, tubular, cylindric (Fig. 52, 3).

Infundibuliform, shape of a funnel, conical (Fig. 52, 4).

Napiform, shape of a turnip (Fig. 52, 2).

Fusiform, outline of a parsnip, narrow at either end, broadest below the surface.

Stratiform, liquefaction extending to the walls of the tube and downward horizontally (Fig. 52, 5).

II. Stroke Culture (see plate cultural characters).

III. Plate Cultures, Colonies:

(A) Form:

Punctiform, dimensions too slight for defining form by naked eye; minute, raised, semi-spherical.

Round, of a more or less circular outline.

Irregular.

Elliptical.

Fusiform, spindle-shaped, tapering at each end.

Cochleate, spiral or twisted like a snail-shell (Fig. 53, A).

Ameboid, very irregular, streaming (Fig. 53, B).

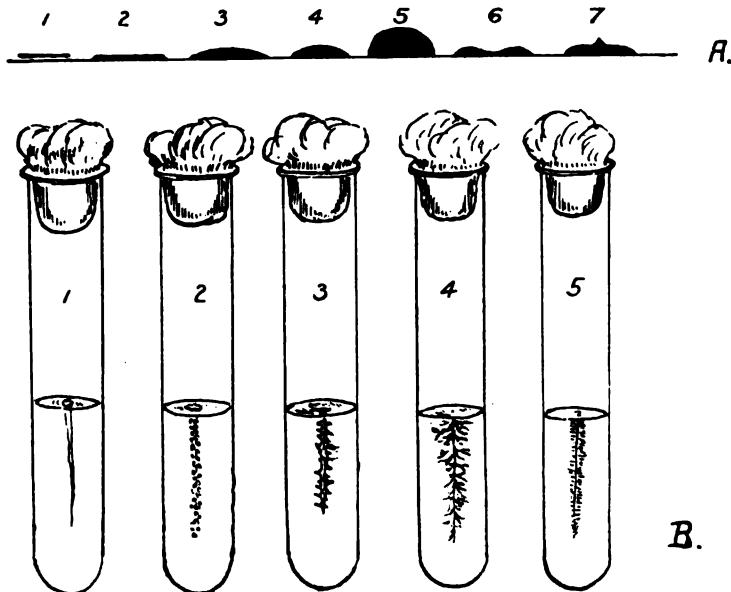


FIG. 51.—GROSS CULTURAL APPEARANCES, NON-LIQUEFYING.—(After Chester.)

A.—Types of elevation of cultures. 1. Flat. 2. Raised. 3. Convex. 4. Pulvinate. 5. Capitate. 6. Umbilicate. 7. Umbonate.

B.—Types of growth along puncture. 1. Filiform. 2. Beaded. 3. Echinate. 4. Arborescent. 5. Villous.

Mycelioid, a filamentous colony, with the radiate character of a mould (Fig. 54, D).

Filamentous, an irregular mass of loosely woven filaments (Fig. 54, E).

Floccose, of a dense wooly structure.

Rhizoid, of an irregular branched, root-like character, as in *Bact. mycoides* (Fig. 53, C).

Conglomerate, an aggregate of colonies of similar size and form (Fig. 55, A).

Toruloid, an aggregate of colonies, like the budding of the yeast plant (Fig. 55, B).

Rosulate, shaped like a rosette.

(B) Surface Elevation:

1. General Character of Surface as a Whole:

Flat, thin, leafy, spreading over the surface (Fig. 51, A 1).

Effused, spread over the surface as a thin veilly layer, more delicate than the preceding.

Raised, growth thick, with abrupt, terraced edges (Fig. 51, A 2).

Convex, surface the segment of a circle, but very flatly convex (Fig. 51, A 3).

Pulvinate, surface the segment of a circle, but decidedly convex (Fig. 51, A 4).

Capitate, surface hemispherical (Fig. 51, A 5).

2. Detailed Characters of Surface:

Smooth, surface even, without any of the following distinctive characters.

Alveolate, marked by depressions separated by thin walls, so as to resemble a honeycomb (Fig. 55, C).

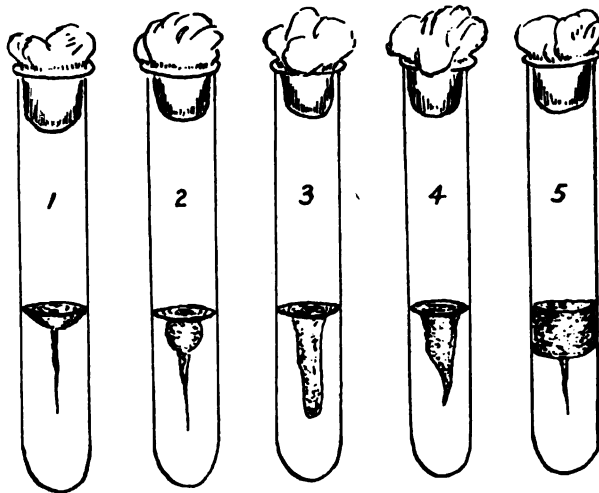


FIG. 52.—TYPES OF LIQUEFYING CULTURES.—(After Chester.)

1. Crateriform. 2. Napiform. 3. Saccate. 4. Infundibuliform. 5. Stratiform.

Punctate, dotted with punctures like pin-pricks.

Bullate, like a blistered surface, rising in convex prominences, rather coarse.

Vesicular, more or less covered with minute vesicles due to gas formation more minute than bullate.

Verrucose, wart-like, bearing wart-like prominences.

Squamose, scaly, covered with scales.

Echinate, beset with pointed prominences.

Papillate, beset with nipple or mamma-like prominences.

Rugose, short, irregular folds, due to shrinkage of surface growth.

Corrugated, in long folds, due to shrinkage.

Contoured, an irregular but smoothly undulating surface, like the surface of a relief map.

Rimose, abounding in chinks, clefts, or cracks.

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(C) Internal Structure of Colony (Microscopic):

1. *Refraction weak*, outline and surface of relief not strongly defined.
2. *Refraction strong*, outline and surface of relief strongly defined; dense, not filamentous colonies.

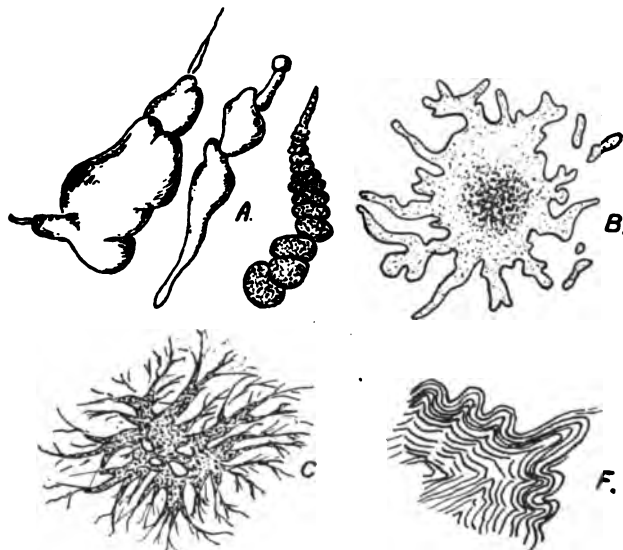


FIG. 53.—SHAPES OF COLONIES.—(After Chester.)

A. Cochleate. B. Ameboid. C. Rhizoid. F. Curled.

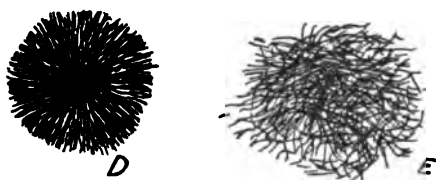
*General:**Amorphous*, without definite structure as below specified.*Hyaline*, clear and colorless.*Homogeneous*, structure uniform throughout all parts of the colony.*Homochromous*, colony uniform throughout in color.

FIG. 54.—SHAPES OF COLONIES.—(After Chester.)

D. Mycelioid. E. Filamentous.

*Granulations or Blotchings:**Finely granular*.*Coarsely granular*.*Grumose*, coarser than the preceding; a clotted appearance; particles in clustered grains (Fig. 55, D).

Moruloid, having the character of a morula, segmented, by which the colony is divided in more or less regular segments (Fig. 55, E).

Clouded, having a pale ground, with ill-defined patches of a deeper tint (Fig. 55, F).

Colony Marking or Striping:

Reticulate, in the form of a network, like the veins of a leaf (Fig. 55, G).

Areolate, divided into rather irregular, or angular, spaces by more or less definite boundaries.

Gyrose, marked by wavy lines, indefinitely placed (Fig. 55, I).

Marmorated, showing faint, irregular stripes, or traversed by vein-like markings, as in marble (Fig. 55, H).

Rivulose, marked by lines, like the rivers of a map.

Rimose, showing chinks, cracks, or clefts.

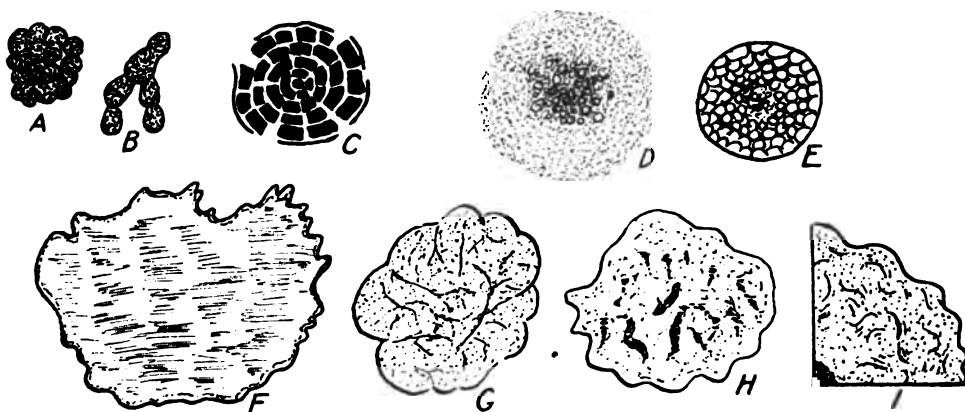


FIG. 55.—SURFACE CHARACTERS OF COLONIES.—(After Chester.)

A. Conglomerate. B. Toruloid. C. Alveolate. D. Grumose. E. Moruloid. F. Clouded. G. Reticulate. H. Marmorated. I. Gyrose.

Filamentous Colonies:

Filamentous, as already defined (Fig. 54, E).

Floccose, composed of filaments, densely placed.

Curled, filaments in parallel strands, like locks or ringlets, as in agar colonies of *B. anthracis* (Fig. 53, F).

(D) Edges of Colonies:

Entire, without toothing or division (Fig. 56, A).

Undulate, wavy (Fig. 56, B).

Repand, like the border of an open umbrella (Fig. 56, C).

Erose, as if gnawed, irregularly toothed (Fig. 56, I).

Lobate (Fig. 56, D).

Lobulate, minutely lobate (Fig. 56, D).

Auriculate, with ear-like lobes (Fig. 56, E).

Lacerate, irregularly cleft, as if torn (Fig. 56, F).

Fimbriate, fringed (Fig. 56, G).

Ciliate, hair-like extensions, radiately placed (Fig. 56, H).

Tufted.

Filamentous, as already defined.

Curled, as already defined.

(E) Optical Characters (after Shuttleworth)

Transparent, transmitting light.

Vitreous, transparent and colorless.

Oleaginous, transparent and yellow; olive to linseed-oil colored.

Resinous, transparent and brown; varnish or resin-colored.

Translucent, faintly transparent.

Porcelaneous, translucent and white.

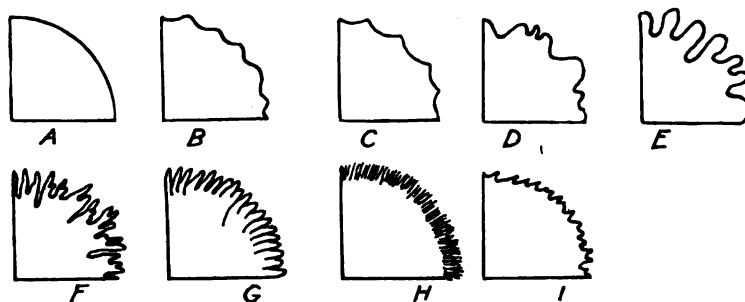


FIG. 56.—CHARACTERS OF EDGES OF COLONIES.—(After Chester.)

A. Entire. B. Undulate. C. Repand. D. Lobate-lobulate. E. Auriculate. F. Lacerate.
G. Fimbriate. H. Ciliate. I. Erode.

Opalescent, translucent, grayish-white by reflected light, smoky brown by transmitted light.

Nacreous, translucent, grayish-white, with pearly luster.

Sebacous, translucent, yellowish or grayish-white.

Butyrous, translucent and yellow.

Ceraceous, translucent, and wax-colored.

Opaque.

Cretaceous, opaque and white, chalky.

Dull, without luster.

Glistening, shining.

Fluorescent.

Iridescent.

LESSON VII.

STUDY OF INDIVIDUAL BACTERIA, THEIR PHYSICAL AND CHEMICAL CHARACTERISTICS.

For the examination of individual bacteria one should make use of a good microscope fitted not only with the ordinary low-magnifying powers (50 to 500 diameters), but also with a clear, flat objective giving with the usual oculars and elongation of the tube an amplification of seven hundred and fifty to one thousand diameters, and with a substage bearing an Abbé light-condensing apparatus (to which an iris diaphragm should be attached for a convenient regulation of light). The student of bacteriologic technique may be supposed to have become familiar, from his previous histologic or biologic work, with the use of the microscope ordinarily, and for this reason no discussion of the principles involved in its construction and manipulation need be entered into here. It may be opportunely suggested, however, that in the examination of fresh, unstained bacteriologic specimens the diaphragm of the light apparatus should be contracted as much as possible consistent with necessary illumination in order to bring into sharp definition the outlines of the microorganisms, but that for stained preparations the field should be flooded with light. The higher objectives (one-tenth or one-twelfth inch focal distance) in common use are oil-immersion lenses, a drop of an oil of refractive index nearly that of the glass of the lens and of the cover and slide (thickened oil of cedar is usually used) being placed on the preparation, into which the tip of the lens dips in focussing, in order to prevent loss of light. The rays would otherwise be diverged on passing from the glass of the preparation into the air between the cover and the lens and would be lost, and the accommodation for light in these short focal distance lenses is so small that all the rays must be conserved for efficiency. The amplification afforded, of course, does not depend only upon the lens, but as well upon the eyepiece of the instrument; but the most definite and clear images are to be obtained with the objectives of short focal distance and oculars of low power and high light capacity. With such an instrument one is enabled to make all of the observations necessary for ordinary bacteriologic investigations; yet there are organisms, as those met by Roux and Nocard in the infectious pleuropneumonia of cattle, which require for their appreciation a much higher magnification than is possible from the lenses suggested (these are barely recognizable with an amplification of two thousand diameters); and the difficulty of recognition of the agencies in yellow fever and other infectious diseases of man makes it quite reasonable to suspect that the failure to detect them heretofore has largely depended on the minute size of the organisms themselves.

When using a low-power lens (one inch or one-half inch focal distance) it is not essential that the material placed upon the slide be covered with the usual cover-slip, all that is essential being that it be arranged in an even moist film through which the light may penetrate with uniformity. So, too, in examination of films which have been

dried and fixed on the slide, a drop of immersion oil may be applied directly to the film without first adjusting a cover-glass if the oil-immersion lens be employed. In all other cases a thin, clear cover is essential for every microscopic preparation.

After using an oil-immersion lens the oil should be removed from the surface of the lens as well as from the preparation by a bit of soft absorbent paper furnished by dealers under the name of "lens paper" (dental absorbent paper). If the oil has become dried upon a preparation a bit of the paper moistened in xylol will be found efficient for its removal; but xylol or any solvent should be used with extreme caution in cleaning an objective lest it remove the balsam settings of the lenses and thus impair the whole apparatus.

Slides and Covers.—The slides made of soft white glass now usually sold by dealers are on the whole the best; but in warm, moist climates they are very liable to corrosion, especially if kept packed closely, and in such localities the green glass regarded as inferior is for common use preferable. Especial attention should be had that the slides are perfectly flat and free from flaw.

Covers should be of white glass, very thin (No. 1 of the manufacturer), and their surfaces true. Squares, five-eighths or three-fourths of an inch in size, will be found most convenient. Like the slides, they should not be kept in close packets lest they become opaque from corrosion.

New slides and covers should be unpacked, soaked for several hours in equal parts of water and one of the strong mineral acids, well rinsed in several changes of clean water, and kept in closed dishes in alcohol to which a little ammonia has been added. Old slides and covers may be easily cleaned after being allowed to soak for some hours or several days in equal parts of strong ammonia and water; after cleansing they should be treated as new slides, being soaked for several hours in a dilute acid well rinsed in clean water, and placed for keeping in dishes of ammoniated alcohol. It is best to keep old slides and covers in dishes separate from those in which are kept the new supply.

MICROSCOPIC EXAMINATION OF CULTURES.

Colonies of bacteria growing in films of the nutrient on plates or in dishes or rolled tubes are to be examined with the low powers of the microscope (twenty to sixty diameters) in order to study their internal structure as to refraction, granulation, markings, etc. (see Lesson VI). For this purpose the plate, dish, or tube is placed directly on the stage of the instrument, and observation made with field darkened as much as permissible, by contraction of the diaphragm; and subsequently reflected light should also be used, the light from the substage mirror being entirely cut off and the rays from some white cloud in the sky concentrated on the surface of the preparation by mirror or bull's-eye condenser. For such examination it is best to have the culture entirely exposed, and for this reason the cover of the Petri dish is removed; but where such exposure is objectionable much may be distinguished through the glass of the cover, or better through the bottom of the dish which has been turned over.

A favorite device used in watching the development of a colony of bacteria and noting its minute features is the "*hanging-drop*" preparation, also employed in high-power studies of the motility, grouping, spore-formation and germination, agglutination, etc., of bacteria. A hanging drop (Fig. 57) requires a special slide, in the upper surface of which a small concavity has been ground and polished. A clean cover-glass having been procured, a drop of physiologic salt solution, bouillon, or of liquefied agar or

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gelatine is placed on the under surface, and with the point of the platinum needle some of the organisms to be studied transferred to the drop. A thin ring of vaseline is drawn about the margin of the concavity in the slide with a hair pencil by which, when the cover is applied, with the drop suspended in the slide space from its under surface, it may be retained in position, drying prevented, and the danger of contamination from the outside removed. When it is intended to observe the development of the colonies of bacteria growing in the inclosed drop, the slide and cover should, of course, have been previously sterilized (in the flame) and the medium used should be gelatine or agar, the whole preparation then being submitted to the usual conditions of temperature and atmosphere (if grown in anaerobic jar, the vaseline ring should not have been complete, to allow the replacement of air in slide chamber with hydrogen or nitrogen) as for any other culture preparation. Where prolonged observation of a selected field is desirable during a period of incubation, special incubators permitting the introduction of the microscope into the interior, and allowing through



FIG. 57.—DIAGRAM OF THE HANGING DROP.

glass sides the entrance of light, are sold; but if one makes the examination in a warm room and uses the mechanical stage for the location of the desired field, this rather cumbersome and costly device is not required. In studies of the individual germs requiring less prolonged observation the loopful of salt solution or bouillon inoculated from the desired growth (or a loopful of an original liquid culture) is usually used instead of the solid media, and there is no need beyond reasonable cleanliness for sterilization. After use of such a slide and cover they should be sterilized and cleansed by one of the methods prescribed for other containers.

Care must always be observed, in the use of the high power with such a preparation, from the danger of breaking the unsupported cover-slip by inadvertent pressure of the lens upon it in focussing. The edge of the hanging drop should be adjusted with the low power, and thereafter the higher power substituted and the edge cautiously focussed, after which it is usually easy, with the fine adjustment and movement of the slide, to obtain the desired field in proper focus.

EXAMINATION OF INDIVIDUAL BACTERIA.

A loopful of physiologic salt solution or bouillon, or even clean water, may be placed on a clean, plain slide, a few bacteria from some culture transferred and diffused by drawing the needle once or twice through the drop (or a loopful of a culture in any of the liquid media may be used instead). A cover is then adjusted and the preparation at once examined (with moderately dark field) under the higher power of the microscope. In such case the arrangement of the individual organisms is, of course, more or less lost, the hanging drop being preferable when the latter feature is sought to be studied. But examination of fresh, unstained material in this manner or by the hanging drop is essential for determination of the motility of the organisms. Neither is comparable to stained preparations for the most exact study of the morphology of the individual germs. In the latter case the material must before being stained be obtained in a thin film ("smear") upon the slide or cover.

Smears for Stained Preparations.—The film may be spread upon either a clean slide or cover-slip, the former being much preferable in that it is more conveniently handled and is in less danger of being broken. The writer usually places the film a little to one side of the center of the slide (not so far as to interfere with centering on the mechanical stage), and is thus relieved of the necessity of using a forceps for handling the preparation. When made on a cover it is essential to use one of the different forms of forceps (cover-glass holder), as that of Stewart (Fig. 58), in order to pursue the work without danger to the preparation or of getting the germs upon the fingers.

The surface of the slide or cover must be entirely clean and quite free from traces of grease which are apt to come on the glass from handling. When taken from the ammoniated alcohol in the tray the slide or cover should be carefully dried with an old linen rag which is quite free from grease from inferior soap or other source, or with a bit of clean, unsized, unfilled paper. A drop of water on the surface should spread out flat and evenly and not separate into small droplets or persist in globular form when it is attempted to draw it over the surface with the needle. Should the drop refuse to spread readily it may be concluded that the glass is not entirely clean; and in such case it is best to wash it with good soap and water, and then rinse it first in water and then in alcohol, and again dry it with paper or a clean rag.

(a) *In examination of cultures* it is preferable to obtain the germs from a growth on a solid medium, the bouillon in a film rendering uniform staining difficult. A small

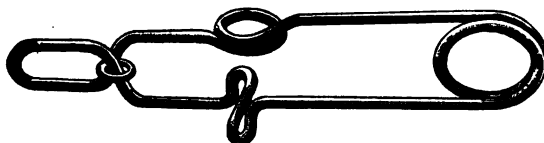


FIG. 58.—STEWART FORCEPS FOR COVER-GLASSES.

drop of clean water is placed in proper position on a slide or cover. The platinum needle, having been flamed, is used to obtain a trace of the culture to be examined, which is then diffused in the drop until a very faint turbidity is noticed (best seen in a side light). With the needle the water with its contained bacteria is now spread over a convenient area, the needle at once flamed to destroy the infection remaining on it and the film allowed to dry in the air. (If a cover was used the film might have been spread by application of a second, clean cover, the two after close adjustment being *slipped* apart so as to evenly distribute the fluid over the adjacent surfaces of the two covers. In this case use forceps in handling.) The drying is completed and the film fixed to the surface of the slide by passing the latter by a slow, even movement through the flame of a Bunsen burner or spirit lamp several times, the film on the upper side and away from the flame, to prevent scorching. Usually, three passages through the flame will suffice; the glass should be distinctly hot, but not sufficiently to be actually painful when touched to the back of the hand. Thus prepared the film should be visible as a delicate opacity, with dead, non-glistening surface. It is now ready for staining or may be kept indefinitely (placed with film upward on a sheet of paper bearing proper label and covered over with suitable cover to keep the atmospheric dust from settling on it—or in a small paper box).

(b) *Smears of Organic Fluids Containing Bacteria.*—Fluids containing organic matter in which bacteria are diffused are made into similar smear preparations by

being added in small proportions to a drop of water on a slide or cover. Thus, one should draw the loop filled with a bouillon or milk culture through a large drop of distilled water in a watch-crystal first; then, having flamed the loop, a small amount of this drop is transferred to the drop of water on the slide and again slightly diffused. The organic matter interferes more or less with the even distribution of the film, and when dried prevents a uniform penetration of the stain; for which reasons the dilution suggested is to be practised. The remaining steps in spreading and fixing the film are the same as above when the material was obtained directly from a mass of bacteria on a solid medium.

The *exudates* and *fluid secretions and excretions* obtained from diseased individuals, as sputum, pus, or urine, are usually spread in films without dilution. When mucus is present in such substance, preventing easy spreading, it is well to warm the slide slightly and insure an even distribution in a thin film by pressing a second slide upon the drop and drawing it across the surface of the first slide. This done, the film is dried in the air and fixed by heat as above. Where, as in case of urine, there is much saline matter present, it is best to remove it before flaming the slide for fixation of the film by placing the slide for a few minutes in a vessel with excess of water, by which these substances will be taken up. It is again dried in the air and the film fixed in the flame. It is then ready for immediate application of the stain, or may be kept as above indefinitely.

(c) *Smears from Tissues*.—After inoculations have been made from foci of disease in tissues obtained by surgical operation or autopsy, a section should be made with a clean knife and the cut surface of the tissue drawn lightly and evenly over the slide. The resulting film is dried in the air and fixed either in the flame or by being immersed for five or ten minutes in a mixture of equal parts of absolute alcohol and ether. It may then be at once stained or kept indefinitely in the fluid or dry. The same procedures are applicable when the material has been obtained on a swab of cotton, as diphtheritic exudate, the swab being wiped lightly over the surface of the slide or cover.

(d) *Impression Films*.—These are prepared by pressing gently and evenly a clean, dry cover or slide to the surface of a colony growing on the surface of some solid medium. After application, it is raised, without sliding, directly from the colony, a print or impression being retained from the adhesion to the glass of the superficial individuals in the growth. As before, the film is dried in the air and fixed in the flame. This procedure is intended for the preservation of the relations of the individual bacteria to each other in the colony, some organisms in their growth producing extremely characteristic arrangements, often in marvelous patterns (*Bact. mycoides*, *vel figurans*).

STAINING REAGENTS AND MIXTURES.

The difficulty of exact appreciation of the isolated bacterium is much diminished by proper staining, for which purpose the basic anilines are most efficient. The ordinary nuclear stains used in histologic work have little penetrative power for bacteria, but if intensely applied are sometimes capable of tinging the bacterial substance. However, their use is attended with such difficulties and the results of such moderate value that they are practically never used, the anilines being employed almost exclusively. Basic fuchsin, gentian violet, methylene-blue, thionine, and a few others are the most commonly used. Aqueous solutions are the most efficient, the dried bacteria in the film preparations permitting their diffusion in the bacterial substance to a far greater

degree than alcoholic solutions, which for the most part are employed only as counter-stains or as stock solutions from which the watery solutions are prepared as required. There is little difference manifested by different types of vegetating bacteria in the assumption of the staining reagents, but some types require, in addition to the simple solution of the dye, the previous or associated influence of substances like the alkalis, aniline oil, acetic acid, or carbolic acid for the proper penetration of the coloring matter. Such a substance is known as a *mordant*. Its precise mode of action is not clearly understood and probably is not in all instances identical. It may involve the removal of some oleaginous material from the bacterial capsule or the production of chemical alterations in the composition of the latter, by which it becomes more pervious to the staining fluid and thus allows the latter to enter the protoplasmic body of the bacterium. The organisms of tuberculosis and leprosy and the mycobacterium of smegma are prominent examples which require the addition of such a mordant for effective staining; and the spores of all bacteria show extreme refractiveness to the penetration of stains, even in the presence of the common mordants.

As a group bacteria are not so readily colored as are animal structures, and in fact there is some difference between different varieties; and, as is the usual rule that such substances as are with difficulty colored may be not readily decolorized, they are apt to retain their coloration in the presence of decolorizing agents longer than associated animal matter, which has been quickly and easily stained. So, too, some difference, available in a few instances for diagnostic purposes, between different types subjected to a definite decolorizer may be distinguished. Of these decolorizing agents water is the type, but the least active; its power of removal of stains is increased when it is used hot. Alcohol is more energetic, a few minutes of exposure of stained films being sufficient to discharge the tint from animal tissues as well as from many bacteria. Among the more powerful decolorizing agents in common use are weak solutions of acetic acid or of the mineral acids.

The process of staining the films as above prepared is usually carried out by placing a few drops of the coloring solution on the film fixed upon the surface of the slide or cover-glass (film side uppermost), contact being continued for from a half minute to several hours in case of different organisms, varying with the type of organism, the staining fluid used, and the application of heat (the process being much shortened when the stain is applied hot). If one prefers, a cover preparation may be floated upon the surface of the staining fluid, the film side downward, in contact with the stain. Everything capable of being colored by the stain having been sufficiently tinged, the preparation is rapidly washed through water or one of the other decolorizing fluids so as to discharge the color from everything in the film but the bacteria. Where several types of organisms of different resistance to decolorizations exist in the preparation, the differentiation is accomplished by permitting sufficient action of the decolorizer to remove the stain from the less resistant, but not sufficient to accomplish the decolorization of the more resistant. As a third step of the process, one may, for greater ease of detection of the stained organisms in the preparation, quickly apply a contrast stain to those elements from which the decolorizing fluid has discharged the first stain. Eosin, vesuvin, methylene-blue, safranin, and Bismarck brown in alcoholic solution are usually chosen for this purpose.

These aniline stains are not so permanent as hematoxylin or carmine stains; preparations are best preserved if kept in closed boxes away from the light.

STAINING SOLUTIONS.

1. There should be kept in the laboratory supplies of the following stock solutions: saturated alcoholic solutions of (a) *basic fuchsin*, (b) *gentian violet*, (c) *methylene-blue*, (d) *thionine*, (e) *eosin*, (f) *resurin*. These are prepared from strong alcohol and an excess of the stain; as used, more alcohol may be added, always with the caution that some excess of undissolved stain remains. Each student has in his locker his individual tray of tubes (Fig. 59), in which watery solutions of the above are kept. One cubic centimeter of the saturated alcoholic solution of aniline dye to ten or twelve cubic centimeters of distilled water are used in the preparation of the latter aqueous solutions. Each tube is provided with tubules as in a chemical wash-flask, for discharge of the stain as needed, a wad of absorbent cotton in the lower end of the exit tubule serving as a filter. Owing to the fact that these watery solutions quickly deteriorate, they should not be used if old, and should never be made up in large quantities.

2. The following special stains and other fluids frequently used in staining are also to be kept in stock:

(a) *Ziehl's Carbol-fuchsin*:

Fuchsin,	1
Absolute alcohol,	10
Five per cent. aqueous sol. carbolic acid,	90

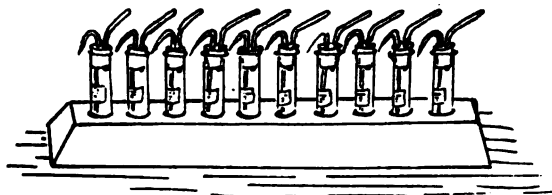


FIG. 59.—TRAY OF STAINING TUBES.

Used in staining mycobacterium of tuberculosis, etc. This solution keeps well for several months. The carbolic acid in its composition is added as a mordant. In preparation throw the fuchsin on a filter paper and after mixing the alcohol and the acid solution percolate until the stain is all dissolved. Filter before use. Contact with films four or five minutes if warm, longer if cold.

(b) *Gabbet's Solution*:

Methylene-blue,	2
Twenty-five per cent. aq. sol. of sulphuric acid,	100

Used as decolorizing agent and as counterstain after carbol-fuchsin. Acid solution should be cooled before being added to the stain. In preparation throw stain on a filter paper and percolate the acid solution until the stain is all dissolved. This solution is not liable to decomposition. Filter before use. Contact with film, cold, for from half a minute to two minutes.

(c) *Loeffler's Blue Solution*:

Sat. alc. sol. methylene-blue,	30
1 : 10,000 aq. sol. potassium hydroxide,	100

Used for general staining, but especially for gonococcus and mycobacterium of diphtheria. This solution is permanent; the alkali serves as mordant. Filter before use. Contact with film from half to several minutes, warm or cold as required in each case.

(d) *Ehrlich's Aniline-water Solution:*

Aniline oil,	5
Distilled water,	100

Shake well the above ingredients in mixture at intervals for five or ten minutes to favor solution of the oil. Filter through a moist filter paper. To each ten cubic centimeters of this "aniline water" add freshly one cubic centimeter of saturated alcoholic solution of gentian violet or fuchsin for use. The aniline water does not keep well and should be renewed in the stock bottle every week or ten days. The mixed stain also keeps poorly and should be made fresh every few days, and filtered before use. These solutions are used as general stains or for special differential purposes in Gram's method; they are useful in demonstration of bacteria in sections of tissues.

In conjunction with these solutions in Gram's method the following decolorizing solution is employed:

(e) *Gram's Differential Solution:*

Iodine,	1
Potassium iodide,	2
Water,	300

Pulverize potassium iodide in a mortar; dissolve in water and add iodine, frequently shaking to accomplish solution. Filter. The solution keeps well.

(f) *Carbol-thionine:*

Thionine,	1
2.5 per cent. aq. sol. carbolic acid,	100

Used as a general stain; valuable as a stain for bacteria in tissues. Add equal proportions of water for staining. Filter before use. Keeps well. Stain three to five minutes. Decolorize with alcohol.

(g) *Unna's Polychrome Blue:*

Methylene-blue,	1
Potassium carbonate,	1
Water,	100

This is used in staining bacteria in tissues. Should be ripened for months before use. Permanent. Contact five or more minutes.

(h) *Acid Alcohol:*

Hydrochloric acid,	3
Seventy per cent. alcohol,	97

This is stronger than the acid alcohol used in histologic work. Used as a decolorizing agent.

(i) *Nitric Acid Solution:*

Nitric acid,	30
Water,	70

Used as a decolorizing agent for *Mycobacterium tuberculosis*, etc.

(k) Acetic Acid Solution:

Acetic acid (glacial),	2
Water,	100

Sometimes used as a mordant, the film preparation being flooded with the solution for half a minute, then washed in water or acid blown off with fine blowpipe. A good decolorizing agent for ordinary stains.

STAINING METHODS.

In the study of any unknown organism it should be a rule to subject film preparations first to the simple aqueous solution of gentian violet or fuchsin, then to Loeffler's blue, to Gram's method, and finally to the Ziehl's carbol-fuchsin and Gabbet's blue, recording the result of each method.

In staining a film with the ordinary aqueous solutions it is customary to flood the film with a few drops of the staining fluid. Usually there is no need of heating; but should the stain be taken up but slowly, the slide or cover may be held over the flame until vapor arises (not boiled). The staining is usually accomplished, when the solution is warmed, in one-half to one minute; when cold, usually from three to five minutes are required. If preferred, the staining solution may be placed in a dish or watch-crystal, and the cover on which the film is spread floated on the surface of the fluid, film side downward. After sufficient exposure to the stain the preparation is held under the tap in a gentle stream of water or waved gently through a dish of water until color is no longer discharged. This is usually sufficient; but should the film retain a deep tint, it is well to wash for a moment in alcohol, and again rinse in water. The film is then dried carefully, being held at some height over the flame until quite dry. It is then to be examined directly in oil, or a cover is added with Canada balsam, as usual, and the preparation then examined with the microscope.

Practically all bacteria may be stained by this method. The mycobacterium of tuberculosis may be stained only with such difficulty, however, that in ordinary preparations containing this organism it is not found. So with tissues containing the *Mycobacterium lepræ*; and the organisms of glanders, Asiatic cholera, and influenza also stain very poorly, but may be demonstrated in deeply stained preparations made with heat.

Loeffler's alkaline blue is of general value, but many of the organisms take up the stain but faintly. It is especially useful for staining the organisms of diphtheria and gonorrhea. The stain is applied by flooding the film with several drops. It acts best if somewhat warmed (not hot), and successful preparations are made in from a few seconds to one or two minutes. The film is then washed well in water, dried, and examined either after application of balsam and cover, or directly in oil (oil-immersion lens).

In the use of Gram's method probably the most important differential staining process is met. For prosecution of this method the film is well stained, with use of moderate warmth, with Ehrlich's aniline-water solution of gentian violet or fuchsin for three or four minutes. The excess of the stain is washed in water, and the film is flooded with Gram's iodine-iodide solution for one minute, the film now becoming dark. It is next washed in ninety-five per cent. alcohol until the color is almost discharged. Should the original tint of the film return persistently, the preparation is again treated with Gram's solution and again washed in alcohol. After almost total

disappearance of the color the film is dried and may be examined in oil (oil-immersion lens) or covered in the usual manner before examination. Counterstaining with vesuvin or eosin may be practised if desired.

The following list includes the most important forms which are decolorized by this method, while the important forms which retain the stain may be inferred from their absence from the group mentioned: *Gonococcus*, *Micrococcus* of Malta fever, *Micrococcus catarrhalis*, *Micrococcus bovis*, *Micrococcus magnus*, *Micrococcus Vincenzii*, *Streptococcus Kirchneri*, *Streptococcus canis*, *Bacterium influenzae*, *Bacterium conjunctivitis*, *Bacterium cancrum*, *Bacterium ægyptiacum*, *Bacterium chinense*, *Bacterium Wrightii*, *Bacterium pneumoniae* (Friedländer), *Bacterium cholerae*, *Bacterium sanguinarum*, *Bacterium avium*, *Bacterium saliva*, *Bacterium ambiguum*, *Bacterium radiatum*, *Bacterium ovatum*, *Bacterium Lepierrei*, *Bacillus Marsiliensis*, *Bacillus coli*, *Bacillus Wardii*, *Bacillus anindolicus*, *Bacillus enteritidis*, *Bacillus chologenes*, *Bacillus toxigenus*, *Bacillus brassicae* (?), *Bacillus icterogenes*, *Bacillus Poelsii*, *Bacillus columbarum*, *Bacillus Breslaviensis*, *Bacillus Salmonii*, *Bacillus levans*, *Bacillus loxiacida*, *Bacillus morbificans*, *Bacillus Silberschmidii*, *Bacillus Murium*, *Bacillus intestinalis*, *Bacillus meningitidis*, *Bacillus typhosus*, *Bacillus pseudotyphosus*, *Bacillus icteroides*, *Bacillus Billingsi*, *Bacillus paradoxus*, *Bacillus pestis*, *Bacillus solitarius*, *Bacillus geminus*, *Bacillus aquatilis-sulcatus-quartus*, *Bacillus primus Fullesii*, *Bacillus tracheiphilus*, *Bacillus pinatus*, *Bacillus Ravanelli*, *Bacillus alcaligenes*, *Bacillus Friedbergensis*, *Bacillus solanacearum*, *Bacillus Weichselbaumi*, *Bacillus phasianii*, *Bacillus Schafferi*, *Bacillus rugosus*, *Bacillus avisepticus*, *Bacillus avium*, *Bacillus meleagris*, *Bacillus tetraonis*, *Bacillus cygneus*, *Bacillus aerobius*, *Bacillus pneumosepticus*, *Bacillus monachæ*, *Bacillus cuniculi*, *Bacillus venosus*, *Bacillus glischrogenus*, *Bacillus albus*, *Bacillus granulatus*, *Bacillus stolonatus*, *Bacillus invisibilis*, *Bacillus venenosus*, *Bacillus murinus*, *Bacillus denitrificans*, *Bacillus Stutzeri*, *Bacillus centropunctatus*, *Bacillus agilis*, *Bacillus Hartlebii*, *Bacillus murisepticus*, *Bacillus Wesenbergii*, *Bacillus larvicida*, *Bacillus dendriticus*, *Bacillus Kornii*, *Bacillus prodigosus*, *Bacillus kiliensis*, *Bacillus licheniformis*, *Bacillus œdematis*, *Bacillus Weigmannii*, *Bacillus saccharobutyricus*, *Bacillus longus*, *Pseudomonas punctata*, *Pseudomonas campestris*, *Pseudomonas pyocyanea* (?), *Pseudomonas capsulata*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Microspira phosphorescens*, *Microspira Schuyllkilliensis*, *Microspira comma*, *Microspira Danubica*, *Microspira Beroliniensis*, *Microspira protea*, *Microspira aquatilis*, *Spirochæta Obermeieri*, *Mycobacterium influenzae*, *Mycobacterium Elmassiani*, *Mycobacterium mallei* (?), *Mycobacterium hastilis*.

Staining of Mycobacterium Tuberculosis and Allied Forms.—The mycobacterium of tuberculosis is especially resistant to staining, and having been once stained is equally resistant to decolorization by means of the mineral acids. In this rests its peculiarity in differential staining. Many bacteria respond nearly or quite equally to this organism when stained by Gram's method, but there are few that are able to withstand the influence of the mineral acids employed for decolorization of the stained preparation. The varieties which approach the tubercular organism are the mycobacterium of leprosy and that of smegma, and several forms of the same group isolated from butter, hay, and grass. The latter very closely simulate the organism of tuberculosis; but excluding these, the degree of resistance to decolorization by acids and by alcohol serves to differentiate the others from the cause of tuberculosis. For demonstration of this microbe the use of one of the aniline dyes reinforced with a mordant is essential, and, with subsequent decolorization of all the elements in the preparation save *Mycobacterium tuberculosis* by one of the mineral acids, affords a

means of clinical diagnosis from the other bacteria apt to be met in persons suspected of having tuberculosis which is practically reliable. A favorite method for determination of these germs in the sputum of consumptives may be detailed in illustration:

1. Spread sputum on a plate of glass over a black surface and with a forceps pick out the small yellowish masses, which are likely to contain numbers of the bacteria. (Do not mistake particles of food!)

2. Place such material on a glass slide and spread by applying a second slide upon the mass, drawing it along and across the slide evenly. (Warm if spreading is interfered with by mucus.)

3. Dry film in air, and fix by passing three times through the flame, smeared side away from flame. (Do not burn!)

4. Flood film with Ziehl's carbol-fuchsin (*supra*, *a*), and warm until vapor arises (do not boil!) over flame for three or four minutes.

5. Wash off excess of stain under tap, and apply as decolorizing agent and counter-stain Gabbet's solution (*b*) for one-half to one minute.

6. Wash in water until no more color is discharged.

7. Dry film and examine directly in oil, or after covering with cover-slip. *Mycobacteria tuberculosis* red; all else in field blue.

The examination of urine and vaginal discharge is similarly carried out, but after discovery of acid-resisting bacteria one should apply a saturated alcoholic solution of methylene-blue to the film for a minute. Should the bacteria be decolorized and stained blue by this procedure they are not the organisms of tuberculosis, but the smegma mycobacteria, non-pathogenic bacteria found in the cheesy secretion about the foreskin or vulva.

Staining Bacteria in Sections of Tissues.—Tissues in which bacteria are to be stained should be at once fixed in absolute alcohol. After sections have been made in one or other of the usual manners (freezing, paraffine, or celloidine methods—for technique of which, consult any work on histologic technology) a section should be attached to a slide. This may be done by floating the section on a drop or two of a solution of gelatine (best sheet gelatine, 0.5; chloral hydrate, 1; distilled water, 100) for several minutes, after which the excess of the solution is drained off and the slide set aside to dry (best until the following day under a bell jar; more quickly at 37° C. in incubator). Thereafter it is plunged for five minutes in a five per cent. solution of potassium bichromate to fix the gelatine glue, and render it insoluble in the staining fluids to be used. (Specimens in paraffine should have paraffine removed prior to this last step.) This done, the staining is carried out as if, instead of a section, the ordinary film were on the slide. It is best, however, to use little or no heat and to allow the stains a longer time for action before removal and application of the decolorizer. So, too, in use of chemical decolorizers the best results usually follow the use of the less concentrated solutions, and separate application of the decolorizer and counterstain. The sections should be almost completely decolorized to gross inspection. In the final dehydration before mounting, absolute alcohol should be used drop by drop on the section, alternating with occasional absorption of all fluid possible by application of a layer of folded unsized paper. Origanum oil is best suited as a clearing agent. After clearing, xylol balsam and a thin cover are to be applied. Carbol-thionine (*f*) is an excellent stain for general use with sections, alcohol being used as the decolorizing reagent; Gram's method and the Ziehl carbol-fuchsin (with Gabbet's solution, if desired) are also entirely appropriate. As a counterstain in Gram's method borax-carmin

may be employed before staining with the aniline-water-gentian-violet solution; or hematoxylin may be used after aniline-water-fuchsin solution.

The above description of staining methods and the necessary reagents is necessarily brief, reference being made to but very few of the many valuable methods applicable to general or special use. For more detail and a more complete list of the methods proposed reference should be made to the standard text-books and systematic works on bacteriology. The special methods of staining spores, flagella, etc., are given in connection with the paragraphs devoted to such bacterial structures.

PHYSICAL CHARACTERISTICS OF BACTERIA.

1. **Shape of Bacteria.**—Bacteria are single-celled organisms. In the unstained preparations, or better after staining, they are seen to present certain shapes, which are in the main characteristic. Uniform persistence of such shape or its variability under altered conditions of growth constitutes an important feature in the *oligomorphism* and *pleomorphism* of bacteria. The oligomorphous varieties (*eubacteria*) are by far the most numerous and include all the known types important in medicine; the pleomorphous, exhibiting a wide possibility of adaptation and growth, are essentially saprophytic and numerically limited and of little except general interest. It is not meant that the shape of a given oligomorphous bacterium is absolutely fixed and invariable, however; certain variations in shape and size being essential in the growth of these as of any germs, and some minor modifications in both of these features and in the physiologic phenomena being manifest from the influence of special conditions of development (*evolutional and involutional* forms). However, such variations are not of such degree as to change the essential type. Three morphologic major types may be distinguished—the *globular* or *short oval* forms, the *long ovals* or *rods*, and the *curved rods*. These served as the basis for the older classifications, the first being known as *cocci* (sing., *coccus*), the second as *bacilli* (sing., *bacillus*), and the third as *spirilla* (sing., *spirillum*); and these terms are constantly used in general application where better definition is not demanded. The modern classifications, while based upon the general outline of bacterial shape, include other features and are much more complicated than such a simple division. In the classification of Migula, adopted with minor modifications in these pages, the first group is included under the family name *Coccaceæ*, the second under the term *Bacteriaceæ*, and the third under that of *Spirillaceæ*. The presence or absence of organs of motility, of definite capsules, of branch-divisions of the bacterial cell, the mode of grouping, and a number of other features are the basis for further division. A rod-shaped organism showing true branching is spoken of as a *mycobacterium*; the *claustridium* is a rod-shaped germ with a thick, clubbed extremity or thick in the middle and tapering at the ends.

These shapes can usually, when marked, be distinguished without special preparation; but for their clear demonstration they are best prepared by one or other of the methods of staining.

Exercise 41.—Prepare two films of cocci from a known culture of *Micrococcus pyogenes aureus*, taking care not to add too much of the growth to the drop of water used in making the film lest the preparation be so crowded that observation with the microscope will be difficult. Stain one film with the ordinary watery solution of gentian violet, without heat, for four or five minutes. Wash well with water. Stain the second film by Gram's

method. Dry and examine in oil without cover. Note the shape of the individual organisms; and after comparison, note the results of use of Gram's stain.

Exercise 42.—From fresh gonorrheal pus repeat the above exercise. Note the gonococci lying in small groups in the cells in the preparation. Note shape of the organisms. Has Gram's method been successful?

Exercise 43.—From a known culture of *Bacillus typhosus* repeat exercise 41. Note the shape of the organisms. Has Gram's stain been successful?

Exercise 44.—From a known culture of *Microspira comma* repeat exercise 41, using as the staining solution for the first film a dilute (1:10) solution of carbol-fuchsin for five or ten minutes with gentle heat. Note the shape of the organisms. Does Gram's method succeed?

2. Grouping of Bacteria.—The occurrence of bacteria in peculiar arrangement is usually the result of incompleteness of separation in the cellular division of the

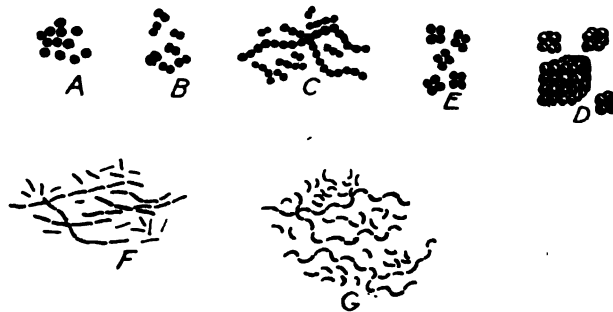


FIG. 60.—GROUPING OF BACTERIA.

A. Micrococci. B. Diplococci. C. Streptococci. D. Sarcinae. E. Tetrads. F. Chains of rods (streptobacilli). G. Chains of curved rods (spirochaetae).

ordinary vegetative mode of reproduction. In multiplication in this manner the cells may divide in one, two, or three planes. In the first case the individuals, if not completely parted, remain placed end to end, chains or filaments being thus produced; in the second, division occurring laterally and longitudinally, films or merismopediae (sing., *merismopedia*) are produced; in the third, division taking place in length, breadth, and thickness, packet forms (*sarcina*) or massive, irregular groups (*zooglae*; sing., *zooglea*) are formed. Such grouping may be often seen in unstained preparations, as in the hanging drop, but best in well-stained films. In staining so as to make clear the delicate lines of division between the individuals of such groups, it is advisable, after having fixed the film by heat, to immerse it for a minute or less in a weak (four per cent.) solution of acetic acid. The best stains are accomplished by very dilute staining solutions left in contact with the film for a prolonged period (one of ordinary stain to four of distilled water, left in contact with the film for ten or fifteen

minutes). Impression films here find their particular use, and should in any doubtful case be resorted to.

From peculiar modes of grouping which are more or less characteristic, these special terms are commonly applied: When cocci occur singly, the individuals are spoken of as *micrococci* (*a*); when in twos, as *diplococci* (*b*); when in chains, as *streptococci* (*c*); in packets of eight or geometric multiples, as *sarcinae* (*d*); when in planes of four, *tetrads* (*e*) (Fig. 60). The term *staphylococcus* was first applied to cocci whose gross colonies presented a fancied resemblance from their lobulated outlines to a bunch of grapes; later, the idea of small microscopic clusters of the individual cocci became attached to the name. The term has no real place in terminology, the same idea being conveyed by the common term "clumps," or zooglea. When rod-shaped forms occur in chains, one usually uses the term *chains* or *threads of bacilli*, etc., although occasionally the prefix "*strepto-*" is applied (e. g., *streptobacilli*). The term *spirochæta* is applied where a single long spiral or spiral chain of short curved elements exists.

Exercise 45.—Examine preparations of exercise 42 carefully for appreciation of the diplococcus shape of the gonorrheal organism; and that of exercise 44, with a view of meeting with spirochæta-like chains of the short comma-shaped individuals.

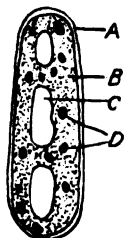


FIG. 61.—STRUCTURE OF A BACTERIUM. — (After Migula.)

A. Cell membrane. B. Protoplasm. C. Vacuole. D. Metachromatic granules.

Exercise 46.—Prepare an impression film from a colony of *Bacillus subtilis*, and a second film of the same organism by the ordinary method. Stain each with ordinary aqueous solution of fuchsin or gentian violet. What differences are apparent?

Exercise 47.—From some sputum from a chronic tuberculous patient with pulmonary cavities prepare a film, and stain with carbol-fuchsin and Gabbet's solution; and examine preparation for tetrads (*Micrococcus tetragenus*); afterward examine the red *Mycobacteria tuberculosis* for branching forms.

3. Structure of Bacteria.—The bacterial cell is composed of a cell membrane, a layer of protoplasm, and a central fluid substance. It is not definitely established that bacteria possess nuclei, but after proper staining there are likely to be found in the protoplasm of the cells certain granules which by some are regarded as of nuclear character. From their coloring reactions they are sometimes spoken of as *metachromatic granules*. They are not ordinarily seen. The cell membrane is generally thin, but in some cases it is thick and not well defined on the outer surface, giving the appearance of a mucous envelope or capsule (whence the class of "*capsule bacteria*"), and is not readily and clearly stained by the ordinary methods. Such capsules, moreover, are not apt to appear in bacteria grown in the ordinary media, but are best seen when the organisms are obtained directly from the animal body or when grown in fluid blood-serum or a few other special laboratory media. The term *ascococci* (sing., *ascococcus*) is applied to the globular forms presenting such encapsulation; in other forms the name *capsule bacteria* is usually used (Fig. 62).

The outer surface of the cell membrane in the majority of bacteria, especially in

the globular and short rod forms, is devoid of appendages; but frequently in the longer rods, both straight and curved, and occasionally in the former, there are to be found delicate hair-like or lash-like extensions known as flagella, regarded as the means of active movement. These flagella are to be seen after the application of special methods of staining, best in young cultures grown upon solid media; and are by no means constantly demonstrable even in the same variety or in all forms of motile bacteria by the



FIG. 62.—TYPES OF CAPSULE BACTERIA.

methods at present employed. However, they are sufficiently constant to be of service in classification and identification (Migula). When such flagellum is single upon the cell, flagellation is said to be *monotrichous*; when numerous at one or both poles of the cell, it is spoken of as *lophotrichous* (flagella polar); when single at each pole, *amphitrichous*; when found all along the sides as well as at the ends the flagellation is said to be *peritrichous*. In the adopted classification coccus forms possessed of flagella are termed *planococci* (sing., *planococcus*); flagellated sarcinae, as *planosarcinae* (sing., *planosarcina*); a straight, rod-shaped organism with polar flagella, as a *pseudomonas* (pl., *pseudomonades* or *pseudomonads*); straight rods with peritrichous flagella, as *bacilli*; straight rods devoid of flagella, as *bacteria*; comma-shaped rods

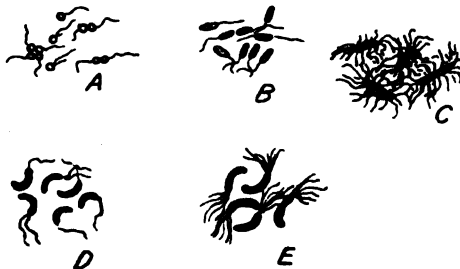


FIG. 63.—FLAGELLATION OF BACTERIA.

A. Planococci. B. Pseudomonads. C. Bacilli. D. Microspiræ. E. Spirilla.

with one or two lophotrichous flagella, as *microspiræ* (sing., *microspira*); comma-shaped rods without flagella, as *spirosomata* (sing., *spirosoma*); the term *spirillum* is retained for those comma-shaped rods which have a bunch of flagella at one or both poles (Fig. 63).

Exercise 48.—Staining of Supposed Nuclear Granules.—Make film from known culture of *Bacillus coli* in ordinary manner; but in fixing pass through the flame ten times instead of two or three. Stain four or five minutes with Loeffler's solution, boiling, replacing the solution on the film as it evaporates. Wash well with water and dry. Examine in oil without cover.

Exercise 49.—Staining capsules (Johns).—Prepare a film of sputum from a case of acute pneumonia, as before described for sputum from a tuberculous case. Cover preparation with two per cent. aqueous solution

of gentian violet and warm until it steams. Wash well in water; moisten with two per cent. acetic acid solution for ten seconds; wash in water; cover, and examine in water with one-seventh inch dry lens. If not successful, remove cover and repeat. If pneumonic sputum be not on hand, use tubercular sputum containing *Micrococcus tetragenus*, which also presents a well-marked capsule.

Exercise 50.—Staining Flagella (Löwit).—Have five slides perfectly free from fat (first washing with soap and water, rinsing in water, then in alcohol and ether, drying with paper; fluid should spread evenly and remain in a thin film, not in drops, if slides be clean). Use young cultures, twenty to twenty-four hours old, on agar, of the following organisms: *Planococcus agilis*, *Pseudomonas pyocyanea*, *Bacillus typhosus*, *Bacillus coli* and *Microspira comma*, for the preparation of films. On each slide place three small drops of distilled water. With usual precautions take from the first culture a bit of the surface of the growth, and with a single, circular, sweeping movement diffuse some of the bacteria in the first drop of the water on one of the prepared slides. Sterilize needle in flame. Take a little of this first drop and in a similar manner diffuse in the second. Flame needle. Repeat diffusion from second to third drop. Dry all three drops in the air and fix films by passing slide through the flame only once or twice, taking care not to overheat. In the same manner prepare and fix films from each of the remaining cultures on the other slides, and in turn stain each preparation as follows: Apply the following freshly prepared mordant, allowing it to act for two or three minutes, cold.

Tannic acid,	5
Distilled water,	20

Dissolve and filter twice. To ten cubic centimeters of the solution add five cubic centimeters of a saturated aqueous solution of copper sulphate, well filtered, and one cubic centimeter of a saturated alcoholic solution of fuchsin. Filter the mixture twice.

After action of this mordant, wash preparation well in water; thereafter stain with aniline-water-gentian-violet for five minutes, cold. Wash well in water and for one or two seconds in fifty per cent. alcohol. Dry and examine as usual. If unsuccessful, repeat. Note the different types of flagellation presented by these organisms. Does *Bacillus typhosus* or *Bacillus coli* exhibit the greater number of peritrichous flagella?

4. Size of Bacteria.—Individual bacteria of the same kind are fairly uniform in size, a feature of some importance in identification. One must recognize, however, a certain variation as occurring in case of the same microorganism, particularly when grown under different conditions and at the beginning of growth as well as in old colonies. The statement of size must, therefore, refer to average individuals, or must

express the extremes of variation. In making measurements one should not be content with but a single observation, but should measure a number of individuals of the preparation (and, too, of the same organism grown under varying conditions), expressing as the result of the investigation either the range of size or the average size of the individuals.

For measurement of such objects the same technique is to be employed as in measuring other minute bodies. A reliable stage micrometer is focussed with the various lenses (no oil to be used when employing the oil-immersion lens), and when proper focus is obtained for each, an eyepiece fitted with micrometer substituted for ordinary ocular. The divisions of the eyepiece micrometer are now calculated in relation with the known measurements of the stage micrometer and carefully recorded lest they be forgotten. The stage micrometer is now put aside and the microscopic preparations of the bacterium substituted and brought into clear focus, the length and thickness for at least ten individuals determined by comparison with the rulings in the eyepiece micrometer, and the limits or the average set down as the result. Thus, one would express the measurements of *Bacillus typhosus* as 0.5–0.8 : 1–3 μ , meaning that it is from five-tenths to eight-tenths of a micromillimeter in thickness and from one to three micromillimeters in length (micromillimeter, one thousandth of a millimeter, one twenty-five-thousandth of an inch). The ordinary bacteria range from somewhat less than one micromillimeter to five or ten micromillimeters in long diameter, but there are organisms much smaller, and others three or four times the larger limit just mentioned.

Exercise 51.—Calculate the value of the spaces in the eyepiece micrometer for the one-twelfth inch oil-immersion lens, and for the one-seventh inch dry lens. Then determine, as instructed, the size of the bacteria of several of the previously stained preparations (e. g., *Micrococcus pyogenes aureus*; *Bacillus typhosus*; *Mycobacterium diphtheriæ*; *Mycobacterium tuberculosis*).

5. Motility of Bacteria.—One of the most striking phenomena to be noted in examining unstained specimens of bacteria suspended in some fluid, as in a hanging drop, is the motion exhibited by the individual cells of many types. (This power of movement may be so persistent that it may remain for some minutes in a dried but poorly fixed film, even after staining and mounting in balsam.) The movements may be very active, darting or undulating in type; or may be slow and scarcely perceptible. One must not, however, confuse the quivering, dancing movement ("Brownian movement") which may appear in case of non-motile bacteria, as well as in case of any inert or dead particles when suspended in a fluid, for true movement; when difficulty is met in distinguishing, one may place the organism in some germicidal solution, as of carbolic acid or corrosive sublimate, when if movement persist it is not due to organic energy. The passive motion induced by currents under the cover-glass, bearing the bacteria, motile and non-motile, in streams in different directions, must not be mistaken for active motion. The means of these active progressive movements were long suspected to be due to the possession of ciliate or flagellate organs before the discovery of such appendages by special methods of demonstration. Even to-day it is impossible from insufficiency of our methods, however, to recognize these organs in a number of motile varieties. In the family of the Beggiatoaceæ a peculiar creeping or waving

movement is noticed, supposed to be due to the presence of an undulating membrane attached to the organism, as in case of the oscillatoria.

To this power of movement must in part be referred the active approach or retreat of motile bacteria to foci of various substances, as points of disease in the body (*chemotaxis*); the similar change of location of non-motile forms probably depending upon growth-progression or passive convection by currents in the surrounding fluids.

Exercise 52.—Rub up a little carmine with distilled water. Arrange a drop as a hanging drop, and examine, noting the dancing movement of the grains. Arrange similarly a drop of water, after diffusing in it a few bacteria from a growth of *Micrococcus pyogenes*. Has it a similar movement? With a needle touch a tiny drop of carbolic acid to the drop and again examine. Does it continue to move? What is the source of movement?

In the same way prepare a hanging drop of *Bacillus typhosus*. Note the movement of the individual rods. How does it differ from that in the previous preparation? Add again a trace of carbolic acid. What effect has it had upon the movement?

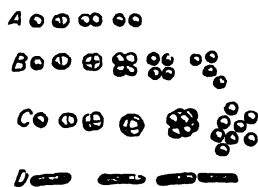


FIG. 64.—REPRODUCTION OF BACTERIA.

A. Division of coccus into diplococci or two cocci. B. Into tetrad or four cocci. C. Into sarcina or eight cocci. D. Division of rod form by transverse separation into two rods.

Exercise 53.—In a similar hanging-drop preparation of *Bacillus typhosus* suspend the smallest possible fragment of sterile, solid agar. Note for fifteen to thirty minutes the approach and attachment of the bacilli to the fragment as illustrative of positive chemotaxis. (Can there be any relation between this experiment and the agglutination phenomenon of the organism?)

6. Reproduction of Bacteria.—Under ordinary circumstances the majority of bacteria multiply as far as is known by direct cell division, a delicate extension of the

cell-wall extending from the sides through the cell-body in one, two, or all three planes. The globular forms may divide in all three planes, but the rod forms are limited to a single, lateral, division. Thus a coccus, before dividing, becomes slightly elongated (Fig. 64) into an oval; and the division occurring in one direction, it is separated into two. These two may not be entirely parted, and remaining together constitute a *diplococcus*. If in the subsequent division of these and their offspring in the same manner the separation of the individuals should not be complete, a chain of *streptococci* results. Sometimes after division in one direction, or coincidentally with it, a second division occurs at right angles to the first plane of division, whence four individuals, or two diplococci, or one *tetrad*, may result. Or, again, a third division at right angles to the other two may take place, whence a *sarcina* form or a *zooglea* of separate individuals may arise. It is estimated that complete division of *Microspira comma* (Asiatic cholera) requires twenty minutes, leading to the possibility of billions of individuals within a single twenty-four hours from an original organism; and it may be said that under fair conditions of growth most bacteria will divide within thirty or forty minutes.

Some bacteria frequently, under comparatively normal circumstances, and many forms under conditions of difficult life (as from unfavorable amount or quality of food, moisture, atmosphere, temperature, etc.), may produce within their substance certain highly refractive, definite bodies known as *spores*, which retain the vital power of the bacteria under adverse states for a long period; and from which subsequently new bacteria of the same type originate. They are thus analogous to the spores or seed bodies of higher types of fungi, but are better thought of as really only resting forms of the original bacterial bodies. They are of importance in the study of the different varieties of bacteria for identification and appreciation of qualities of vital persistence.

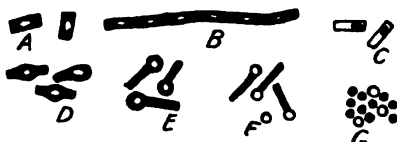


FIG. 65.—TYPES OF SPORULATION.
A, B, C, D, and E. Endospores. F and G. Exospores. A, B, D. Spores equatorial. C and E. Spores polar. F. Arthrospores.

Such spores, while probably always truly within the bacterial body in their formation (hence *endospores*), are sometimes seen free (*exospores*) in the midst of adult bacteria, or attached to the ends of the bacterial cells (*arthrospores*), the envelope of the original bacterial cell having disappeared from about the spore. It is a rule that no more than a single spore is formed from one bacterium; from the position in which it occurs it is described

as *equatorial* or *polar* (Fig. 65); and the cell outline is apt therefrom to be swollen into *claustridium* or *club* shape.

For the development of these spores into the ordinary vegetative forms there must be afforded proper conditions of life, particularly plenty of moisture, through the imbibition of which apparently the bacterium is enabled to grow and force its mass through the capsule of the spore. This is spoken of as *germination of the spore*; it may take place at one or both poles or along the sides, and is hence described as *polar*, *bipolar*, or *equatorial germination* (Fig. 66). Usually spores are met among the rod-shaped organisms, but the globular forms are now and again also capable of spore



FIG. 66.—FORMS OF BACILLI SHOWING SPORES.

formation. They are often easily seen in ordinarily stained preparations as rounded, or oval bodies within, or among, or apparently on the ends of the ordinary bacteria, unstained and peculiarly shining. Their refraction is especially appreciated when the field is darkened by contraction of the diaphragm of the substage. By prolonged staining with the application of considerable heat and the use of a mordant in the staining fluid, they may be colored; after which they are usually with more difficulty decolorized than the ordinary bacteria, which makes possible their differentiation. The following method has been found very satisfactory for their demonstration (modified from Hauser): A drop of water is placed on a slide and in it diffused a small amount of a sporulating culture of some organism. The drop is spread and nearly

dried in the air. Before it is quite dry, the film is flooded with carbol-fuchsin solution, which is heated nearly to boiling and evaporated; the slide with the evaporated stain being passed several times through the flame to fix the film. More of the stain is now added and boiled for five minutes, fresh solution being furnished as evaporation takes place. At the end of this time the excess of stain is poured off and the film well washed in hot water until the color is discharged from the organisms (but not from the spores). This usually requires several minutes; and it is well from time to time to drop a cover on the wet film and examine the preparation with the one-seventh inch lens to determine when to refrain from further decolorization. This accomplished, the film is stained for a few seconds with Loeffler's blue, washed in cold water, dried, and examined. The spores will be found red, the bacteria blue.

In addition to the possibility of determining the presence of spores by actual microscopic observation, it may be inferred that they are present (and further search in microscopic preparations should then be made) if after exposing a culture for ten minutes to 80° C. inoculations made from it upon fresh medium are followed by growth. In this the adult forms of bacteria are destroyed by the heat exposure and only spores are likely to persist in their vital condition, further growth depending upon their germination.

It is believed that a third mode of reproduction occurs, at least in the higher forms, as the mycobacteria, from segmentation of the rods into small coccoid bodies, analogous to the *gonidia* of the hyphomycetes, and spoken of as such in this connection. They are small, not so refractive as spores; and, like spores, they are believed to be very resistant to adverse conditions of life. Under favorable conditions they develop by simple growth into the adult types.

Exercise 54.—From an agar culture of *Bacillus subtilis*, grown in the incubator for several days, one can usually obtain numerous spores or sporulating individuals; often nearly the whole preparation seems made up of these to the exclusion of the ordinary rods. Prepare and stain a film from such source according to the above instruction. What is the position of the spores in the bacilli?

Exercise 55.—In a few drops of sterile bouillon place a number of spores obtained from culture used in previous exercise, and keep in incubator. Every ten minutes for an hour or more prepare films, staining them quickly with Loeffler's solution, warm. The spores will be unstained, the germinating organisms blue. Is germination equatorial, polar, or bipolar?

CHEMICAL ACTIVITIES OF BACTERIA.

In the growth of bacteria, both in the living host and upon laboratory media, it is well known that important metabolic changes proceed in the bacterial cells, as well as chemical alterations in the medium on which they develop. Little is known of these processes, but in connection with them a number of phenomena, as production of pigment, light, heat, various types of fermentation and putrefaction, and other manifestations, take place along with the growth, multiplication, and death of the bacterial cell. The demonstration of such changes is in a large measure favored or prevented by the character of the medium upon which the bacteria are grown; and

the precise relationship of any bacterium to a given standard culture medium must in the study of these chemical phenomena be recorded to give any observations their full value for the classification of the organism.

1. **Pigment Production.**—Various pigments, probably analogous to the coloring principles of higher plants, are developed by a number of bacteria (known as *chromogenic bacteria*). These coloring substances are in some instances probably retained within the bacterial body; in others, are diffused more or less in the medium of growth. They are of varied hues—yellow, red, blue, violet, black, green, fluorescent (reflecting colors different from the hue of the culture itself), or iridescent (manifesting a play of colors as of a rainbow). Little is known of these pigments other than the few peculiarities mentioned and their solubility in different reagents, as water, alkaline or acid solutions, carbon disulphide, alcohol, ether or chloroform. They are of complex organic composition, iron being probably a more or less important factor in most, its absolute absence from the medium of growth being usually followed by failure of chromogenesis. They are best seen in cultures grown in media containing carbohydrates, as potato. In the ordinary bacteria the coloring matter is not visible in the individual cells as examined with the microscope; there are a few instances, apparently approaching the lower algæ, in which a slight green tint is to be noticed, perhaps of the nature of chlorophyll; and in the *thiobacteria* and *rhodobacteria*, granules of a reddish or violet color are found (sulphur or bacterio-purpurin).

Note.—Here should be demonstrated a number of the common types of chromogens in gross colony.

Exercise 56.—Grow *Bacillus prodigiosus* upon potato at room temperature, and another similar culture at 37° C. Note the red color of the first and the absence of color from the second. Likewise plant this organism on agar free from sugar and carry it through several generations to observe loss of chromogenesis.

Exercise 57.—Plant *Pseudomonas pyocyanea* upon two tubes of agar. Close one of these tubes with rubber stopper (or put in anaerobic jar), leaving the other protected only by the cotton plug. Grow in incubator and compare appearance of growth at close of each twenty-four hours for several days.

Pour into the second tube, showing a beautiful blue-green color diffused through the agar, a little chloroform; allow it to stand for some minutes and observe the solution in the fluid of the blue color (*pyocyanin*). In a potato tube of the same organism note the yellowish-brown color of the culture. Press upon the surface of the growth with the sterilized platinum needle, and observe that in a few minutes a green color has succeeded the brown (*chameleon reaction* of *Pseudomonas pyocyanea*), later fading back to the original brown tint.

2. **Photogenic Power.**—The phenomenon of light production belongs to a small group of bacteria and is apparently a characteristic of the vital activities of the germs rather than due to any chemical product of the organisms. The light is of course not visible in the lighted room, but is apparent at night or in a darkened room; and

is of a pale, phosphorescent type. Like pigment production, it is interrupted or modified in intensity by conditions altering the vital activities of the bacteria, as temperature, atmospheric relations, and various chemical agencies, and the like. Photogenic bacteria are apt to be encountered in cultures obtained from salt water or salt fish; and photogenesis is most apparent and best preserved when such bacteria are grown in media rich in saline elements (as nutrient gelatine made from an infusion of salt fish in natural or artificial sea-water, with addition of one per cent. of peptone, one per cent. of glycerine, and 0.5 per cent. of asparagin—Neumann and Lehmann).

Exercise 58.—Let the instructor here demonstrate in the dark-room the light production of one of the phosphorescent bacteria grown on above medium.

3. Ferments.—By fermentation is meant the splitting up of complex organic molecules into simpler ones through the agency of a so-called *enzyme*, or non-living ferment, which is itself not destroyed in the process it excites. Such enzymes may operate in the presence of various substances which are harmful to the bacteria themselves, and are found active in the germ-free filtrate from cultures; and are therefore to be regarded as not residing in the bacterial cells, but rather as a product of elimination from the cells.

(a) *Proteolytic (Albumin-dissolving) Ferments.*—These are present in the cultures of a large number of bacteria, and give rise to the common phenomenon of liquefaction of gelatine media (the glue in gelatine) and blood-serum. The production of peptones or propeptones is a result of such liquefaction. It may be demonstrated by a thorough filtration of a liquefied gelatine culture through a porcelain filter, adding to ten cubic centimeters of the filtrate 5 grams of ammonium sulphate and keeping it warm for half an hour (thus precipitating all albumins but the peptones and propeptones), then refiltering and testing the latter filtrate with an alkaline solution of cupric sulphate, when the peptones strike with the reagent a violet color.

Exercise 59.—Plant in a flask, or a number of tubes, of gelatine medium *Bacillus prodigiosus*, and allow the material to become completely liquefied. Then through a porcelain filter sterilized in the autoclave separate the bacteria from the liquid holding soluble albumins in solution. Divide the latter into two parts. To one part (10 cubic centimeters, at least) add an excess of ammonium sulphate, which will precipitate all of the albumins but the peptones and propeptones. Refilter and test the filtrate with Fehling's solution of copper sulphate for the violet reaction of peptones.

Add the second part of the original filtrate (free from bacteria) to a fresh tube of gelatine; observe that in the course of twenty-four to forty-eight hours liquefaction of the gelatine takes place from the action of the bacteria-free enzyme.

Plant a tube of gelatine each from known cultures of *Micrococcus pyogenes*, *Bacillus coli*, *Bacillus typhosus*, *Mycobacterium diphtheriæ*, and *Microspira comma*. Note the results as to liquefaction in each case.

(b) *Diastasic Ferments*.—A number of bacteria possess the power of converting starch into glucose by means of an elaborated diastasic enzyme.

Exercise 60.—Inoculate several tubes of sugar-free bouillon (or peptone solution) with *Bacillus subtilis* and incubate for eight or ten days. Prepare a thin starch paste, adding two per cent. of thymol (which should be tested for sugar previously, to insure the absence of the latter), and mix equal parts of the culture and paste. Put aside for six or eight hours in the incubator and then test with Fehling's solution for the presence of glucose.

(c) *Invertin Ferments*.—Such ferments are developed by a few bacteria, and accomplish the transformation of cane-sugar into glucose.

Exercise 61.—Make a ten days old culture of *Microspira comma* in sugar-free bouillon. Prepare a two per cent. solution of cane-sugar, adding two per cent. of strong carbolic acid. Mix equal parts of the culture and sugar solution. The carbolic acid should restrict the bacterial activities, but will not prevent the action of the enzyme; and if the mixture be allowed to stand for several hours and then tested with Fehling's solution, the glucose reaction will be obtained. As a control, test in the same manner the cane-sugar and carbolic acid solution, noting the failure of the copper reduction.

(d) *Rennet Ferments*.—The phenomenon of milk coagulation may be dependent upon the lactic acid produced from the milk-sugar in a culture of a given organism, or to an enzyme. To demonstrate the latter a culture of some bacterium incapable of such acid formation is to be selected, as *Bacillus prodigiosus*.

Exercise 62.—A culture of *Bacillus prodigiosus* upon milk is prepared and placed in the incubator for twenty-four hours, when without change in color the milk will be found solidly coagulated. What has been the influence as to reaction of the medium?

Or, a sugar-free bouillon culture of the organism is prepared (ten days old) and filtered through a porcelain filter, equal parts of the filtrate and of a milk tube mixed and placed in incubator temperature for a few hours, when the same result is reached.

Plant in milk from known cultures of *Micrococcus pyogenes*, *Bacillus typhosus*, *Bacillus coli*, *Mycobacterium diphtheriæ*, and *Microspira comma*. Note results in each case.

A number of other types of fermentation are commonly met with, as the alcoholic fermentation of sugar, that of the production of acids, as lactic acid or acetic acid from sugar and alcohol; the production of alkaline substances, as ammonia, from proteids free from sugar (*urea-fermentation*) by various bacteria. It is claimed that free acid is formed only in sugar-containing media; and upon the supposition of the early splitting of the small amount of meat-sugar in ordinary media or in media to

which sugar in small proportions has been added, is explained the frequently observed phenomenon of an early acid reaction in such growths, followed later and overcome to an alkaline reaction by the alkaline substances produced in the splitting of the nitrogenous molecules into ammoniacal products. These fermentations are probably also due to enzymes. It seems probable that in the ordinary alcoholic fermentation this enzyme is retained in the bodies of the ferment cells. Such an enzyme by high pressure has been obtained from yeast cells and is known as *zymase*; and it seems likely that a similar or identical substance exists in bacterial cells capable of the same process. In effect, in such processes the fermentescible substance probably goes through the bacterial body and the process is closely related to metabolism. It probably is performed for acquirement of some of the energy (heat) set free in the splitting process. Closely related to these last considerations is the power of

4. Gas Production.—A number of gases may be produced by various bacteria in different media. In the sugar-containing media in the conversion of the sugar molecule into alcohol and carbon dioxide, the latter, as well perhaps as a few other gases, as hydrogen and methane, are produced; and in the albuminous media ammoniacal gases and sulphuretted hydrogen may be encountered. For the purpose of observation of gas production solid media may be utilized, note being taken of gas bubbles produced in the media which have been inoculated by bacteria capable of generating such gases. Or liquid media in the fermentation tubes described in a previous section may be employed.

Exercise 63.—A fermentation tube is filled without bubbles with a bouillon containing one per cent. of glucose or other sugar, sterilized in the autoclave, and after cooling is inoculated with *Bacillus coli* and put aside for twenty-four to forty-eight hours in the incubator, when gas will be found to have collected in the closed end of the tube. This gas is largely composed of carbon dioxide from sugar destruction. There is usually active sulphuretted hydrogen formation as well. To recognize the latter, the following procedure may be practised. A slip of paper moistened with a solution of acetate of lead is fixed in the bulb of the tube alongside of the cotton plug and a dark rubber cap (free from sulphur) tightly fitted over the opening. By tilting the tube the gas is now permitted to pass to the bulb, and after allowing it to stand for a time, the lead will be found blackened into the sulphide.

If now in a similar tube the amount be marked by wax-pencil mark on the glass, and the bulb be filled with a ten per cent. solution of sodium hydrate, the thumb being tightly placed over the opening and the tube inverted and shaken so as to bring the soda solution and the gas together, and the gas thereafter returned to the closed end of the tube, the amount will be found diminished. This loss represents the proportionate amount of carbon dioxide which was present, and which has been absorbed by the sodium hydrate. In determining the amount of gas generated in these tubes proper corrections should be made for atmospheric pressure and

temperature; or at least the conditions of pressure and temperature noted in the records. Moreover, daily records should be kept, and the amount accepted as final only four or five days after gas production has ceased. Repeat with *Micrococcus pyogenes*, *Bacillus subtilis*, *Bacillus typhosus*, *Microspira comma*, and *Mycobacterium diphtheriæ*.

5. Acid Production.—It is asserted that free acids are produced only in carbohydrate-holding media, and are producing in sugar-holding media either from the sugar directly or from the alcohol after alcoholic fermentation. Of these acids, lactic acid is the most prominent, but there may also be traces of acetic acid, butyric acid, and a few others. The actual analysis of the media for such acids is too complicated for discussion in this place, and the mere demonstration of an acid change in the reaction of the medium must be sufficient.

Exercise 64.—To neutral bouillon is added a drop or two of alcoholic solution of rosolic acid, so as to produce a distinct pink color. Inoculate the tube with *Bacillus coli* and place in the incubator. Note that for a time the color is diminished, indicating the production of an acid reaction; after a few days, however, owing to exhaustion of sugar, this ceases, and from the production of ammoniacal compounds the reaction changes to alkaline, causing the color to reappear and grow more distinct.

Repeat with cultures of *Micrococcus pyogenes*, *Bacillus subtilis*, *Bacillus typhosus*, *Mycobacterium diphtheriæ*, and *Microspira comma*.

Exercise 65.—To sterilized milk a little sterile litmus solution is added so as to cause the medium to become slightly blue. To such preparation is added a loopful of *Bacillus coli*, and it is allowed to remain in the incubator for a time. At first the blue color changes to red, the latter later disappearing and a deeper blue assumed from the alkaline change. The milk-sugar at first was changed to lactic acid; when the sugar was used up, the proteids were progressively changed into alkaline compounds.

6. Alkaline Production.—This, as already referred to, is believed to be due to the splitting of nitrogen compounds in the medium employed by certain bacteria in a manner of the same type if not identical to metabolism; and the products as far as known are ammonia, amine, and ammonium bases. It is the same process as is concerned in the so-called urea-fermentation, by which the urea of fresh urine is converted into ammonium carbonate, from which other ammonium bases may thereafter develop, as ammonium urate or phosphate. To exhibit this alkaline production, which is common to a large number of bacteria grown on sugar-free proteids, the following will serve:

Exercise 66.—*Bacillus coli* is inoculated into a tube of peptone solution and grown at body temperature or lower (20 to 37° C.), for from ten to fourteen days. The medium should have been exactly neutral to phenolphthalein, or if this has not been used, a fresh tube of the same reaction as

that employed used as a control. A drop of one per cent. alcoholic solution of phenolphthalein should be used as an indicator, which when added to the inoculated tube at once strikes the usual red color of an alkali. If the medium was not exactly neutral to phenolphthalein when the organism was introduced, the same amount of the indicator should be added to the control tube and the intensity of color in the former compared with that in the latter tube.

Repeat with cultures of *Micrococcus pyogenes*, *Bacillus subtilis*, *Bacillus typhosus*, *Mycobacterium diphtheriæ*, and *Microspira comma*.

7. Alkaloidal Products.—Among the products of bacterial activity there is a large group (a) of basic nitrogenous bodies, known collectively as *ptomaines*, belonging to the *amines* ($\text{N}^{\text{H}}\text{—CH}_2$), as cadaverine ($\text{N}(\text{CH}_2)_5\text{C}_3\text{H}_5\text{OH}$), sepsine and putrescine; *ammonium bases*, as choline, neuridine, muscarine, etc.; *pyridine derivatives* (derived from pyridine, $\text{C}_5\text{H}_5\text{N}$), as collidine ($\text{C}_8\text{H}_{11}\text{N}$), indol ($\text{C}_8\text{H}_7\text{N}$), and skatol ($\text{C}_9\text{H}_9\text{N}$); as well as *amido-acids* (as leucine, tyrosine, etc.), and others.

The method of isolation of these bodies is for the most part too complex for the limits of the present outline. Generally speaking, these are not the seriously poisonous products of bacteria.

(b) *Toxalbumins*.—In this group occur a number of the essential toxins or poisons of pathogenic bacteria, *bacterioprotoins* (as the old form of tuberculin and mallein), and *bacterioplasmins* (poisonous principles extracted from a number of pathogenic organisms, as of cholera or typhoid fever, by pressure). The toxins or toxalbumins proper are substances which may be precipitated as amorphous poisons from bouillon cultures of various bacteria (as *Mycobacterium diphtheriæ*) by the ordinary albumin-precipitating reagents, as by alcohol. These substances are extremely unstable and easily destroyed by heat, chemicals, exposure to light, and other agencies. They are the most important, from a pathogenic standpoint, of all the bacterial poisons and are responsible for many of the specific symptoms of infectious diseases; and in most cases are active in extremely small amounts. There is some belief that instead of true albumins in these bodies, we are dealing only with certain unrecognized bodies in the midst of the albumins of the medium carried down in precipitation.

Bacterioprotoins differ from these latter in that, aside from their smaller toxic power, they are not altered by heat.

8. Antitoxins.—In this general connection should be mentioned, briefly because of our meager knowledge of their nature, the protective substances which exist or are developed in the fluids and tissues of the animal body influenced by an infection. These, of course, are not substances to be regarded as direct bacterial products, but for convenience may be considered here in outline. The protection or immunity of an individual from infections depends either upon some mechanical protection (as that of the skin and other protective coverings, or of phagocytosis) or upon certain chemical antagonisms (as the acids or alkalies in the secretions with which the infection is brought into relation, or the alexins and antitoxins). *Alexins* are protective substances naturally pre-existing in the fluids of the body (serum and lymph), supposed to be derived from the leucocytes. They have not been isolated, but are known to be very unstable to heat (55°C . or above) and sunlight. They possibly aid phagocytosis by killing bacteria, after which the leucocytes may more efficiently destroy

and remove them. *Antitoxins* are substances which are developed mainly in the blood-serum during the course of an infection, which have antagonistic influences upon the toxins of disease (either *passively*, as when before the introduction of a toxin into an animal body it may be uniformly rendered innocuous by mixture with serum containing the antitoxin, in which action the latter may be thought of as distinctly antidotal and protective; or *actively*, where after the introduction of an infection into the animal its effects are overcome by introduction of a certain amount of the antitoxic serum, although a mixture of the same proportions of toxin and antitoxin outside the body and its introduction into a smaller animal are followed by the usual effects of the infection. In this latter form the antitoxin apparently stimulates some elements of the infected body to a direct antagonism—or is, in other words, healing in its influence).

9. Agglutination Phenomenon.—In this same connection may be considered the agglutinating phenomenon of serums of persons infected or recently well of various diseases, as typhoid fever, over the bacteria of the same specific disease. This manifestation depends upon some directly bactericidal substance (contrasting with the antitoxins which antagonize rather the poisons of the bacteria in the serum); in some instances actual bacterial destruction (*bacteriolysis*) may take place, while in others rather a paralyzant influence is exerted upon the organisms as is seen in the stoppage of movement of the germs. Where such bactericidal substances are powerful and persistent in the serum of the patient, the immunity granted to the individual is likewise persistent. The nature of these substances is not understood.

Exercise 67.—After the tenth day of the disease in typhoid fever, a finger is well cleansed and dried and pricked so that several large drops of blood may escape. These are caught in a clean glass tube, as in an ordinary dropper or in a capillary tube. This blood is allowed to clot and the serum to separate (if a capillary U-tube is used it may be centrifugated for the separation).

A twenty-four-hour bouillon culture of the typhoid bacillus is made. With a capillary pipette of the same caliber as that used for the collection of the serum a suitable amount of the culture is withdrawn. (Care is to be taken to plug the upper end of the pipette with cotton to prevent accidental suction of the culture into the mouth of the operator.) A certain length of the serum tube is now broken off (after filing) and its contents blown out into a watch-crystal (one centimeter length broken off, and serum expelled by holding the broken part in forceps and blowing it out with another fine tube inserted in end). Fifty times this amount (ten centimeters length taken five times) of the bouillon culture are added to the serum, mixed, and a drop placed on the slide and covered (hanging drop may well be made). These proportions vary with different observers, as weak a dilution as one to ten having been originally practised. At the close of varying periods, ranging from fifteen minutes to as much as two hours, according to various directions, the typhoid bacilli will be found to have ceased from active movement and to have agglutinated in clumps. Extreme limits of

time should be avoided in practice, and the reaction accounted positive only when occurring within the first hour; if strong, it will probably have occurred within a few seconds. Should failure occur with one to fifty dilution, a one in twenty-five dilution may be tried, and failures with this may be accounted negative. If very strong, bacteriolysis of the typhoid germs may also be noted, the destruction beginning with vacuolization and erosion of the bacteria.

The same result may often be obtained in a tube by the addition of large amounts (in same proportions as above) of the serum, the agglutinated masses forming a flocculent precipitate visible to the unaided eye, and the previously turbid bouillon becoming clear between the tiny flakes. In the course of twenty-four hours the effect of agglutination and stoppage of motion will usually be found to have disappeared, the agglutinating agent having apparently been exhausted from the serum employed.

10. Indol and Phenol Production.—Among the ptomaines *indol* and *phenol* are substances of importance in that they are demonstrable with little difficulty, and, being more or less characteristic of certain organisms, serve to aid in their identification. Aside from the last feature it is not known that these substances are of importance.

Indol occurs particularly in the old cultures of bacteria of the colon group and of the various microspiræ and spirilla. It is also found in old cultures of *Mycobacterium diphtheriæ* and *bacterium mallei*. It is shown by adding to a tube of bouillon free from sugar (or the ordinary peptone solution), in which one of the indol-producers has been grown for eight to ten days, an equal amount of weak nitric or sulphuric acid (twenty per cent.). In case the germ has also produced nitrites in the medium, or if nitrites be present in the nitric acid used, the reaction, consisting in the production of a red color (probably due to the formation of nitroso-indol nitrate), occurs. But in the absence of the nitrites it will be necessary to add a drop of a one per cent. solution of sodium or other nitrite. Should the reaction take place without the addition of nitrite, upon the addition of sulphuric acid alone, it may be tentatively inferred that the organism is also a nitrite-producer.

Exercise 68.—Grow in sugar-free bouillon or peptone solution a culture of *Bacillus coli* for eight or ten days. To this culture add an equal amount of twenty per cent. pure sulphuric acid, and if needed slightly warm for a few minutes. Now add a drop of a one per cent. solution of sodium nitrite and continue to add and warm until the color is at its intensity. Too great an addition of nitrite will cause a yellowish tint to be assumed.

Repeat with cultures of *Micrococcus pyogenes*, *Bacillus subtilis*, *Bacillus typhosus*, and *Mycobacterium diphtheriæ*.

Exercise 69.—To a similar culture of the cholera organism add the acid alone, the color being produced without the addition of the nitrite, showing the production of the nitrite as well as of indol by this bacterium. The

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reaction has been long known in connection with this organism as the "cholera red reaction."

Phenol production occurs with much the same groups of organisms as that of indol. For its demonstration it is essential to use a larger quantity of growth in sugar-free bouillon than in case of effort to demonstrate indol presence. To this is added one-fifth its volume of pure hydrochloric acid and the mixture distilled. The distillate contains the phenol, which, after neutralization of the distillate with calcium carbonate, will strike a violet color upon the addition of neutral solution of chloride of iron (one per cent.).

Exercise 70.—Make a culture of *Bacillus coli* in sugar-free bouillon or peptone solution, using about fifty cubic centimeters of the medium. After eight or ten days add ten cubic centimeters of hydrochloric acid, and in a condenser (or retort) distil five or ten cubic centimeters from the mixture. Carefully neutralize with powdered calcium carbonate (or calcined magnesia), and add drop by drop a one per cent. solution of ferric chloride to obtain the violet color produced by phenol. A white flocculent precipitate may be produced in a separate part of the distillate upon the addition of bromine.

11. Nitrifying and Denitrifying Bacteria.—Quite a large number of bacteria apparently possess the power of forming nitrite in cultures, although it is not clear how this is accomplished; and possibly, when it is found in traces only, it may have been absorbed from the air. In the study of indol formation the lack of need of adding nitrite to bring out the characteristic reaction in many examples is probable evidence of its presence. As far as is known, certain pseudomonads found in the soil (*Ps. Europa*, *Ps. Javanensis*) are alone able to convert, the first the nitrogen of ammonium compounds into nitrite, the second nitrite into nitrate. A number of bacteria found especially about the roots of certain plants (*e. g.*, leguminosæ) apparently absorb the nitrogen of the air, preserving it in the soil as free nitrogen or transforming it into nitrites or into ammonium. The general term *nitrifying bacteria* is applied to the forms engaged in the above processes; these are of much importance to the agriculturist in their rôle in enrichment of the soil.

On the other hand, many bacteria possess the property of reducing nitrates to nitrites by the abstraction of oxygen, or in the same way of reducing the nitrites to free nitrogen, sometimes leading to the formation of ammonium. These are termed the *denitrifying bacteria*. They are widely distributed in dung, soil, and putrefying organic matter, and the reduction of nitrates to nitrites seems common to the majority of bacteria. The reduction of nitrates to nitrites is usually determined by growing a bacterium in question for seven days either in the room or incubator temperature, as is best fitted, in a medium made up of water with but a small amount of peptone added and with a little saltpeter (tap-water 1000, 1 gram dried peptone, 0.2 gram sodium nitrate). To two tubes containing about three cubic centimeters, each thus prepared, inoculated and grown, and to a control tube of the same size and same content of the saltpeter broth and kept in the same conditions as the inoculated tubes, is added a mixture of naphthylamine and sulphilic acid (naphthylamine 1, distilled water

1000—kept in clean glass-stoppered bottle; sulphanilic acid 0.5 gram, dilute [1:16] acetic acid 150 cubic centimeters—also kept separate in glass-stoppered bottle. Mix equal quantities of the two solutions for use. Add to first inoculated tube and control tube two cubic centimeters each of the mixture). The tubes are closed with rubber stoppers and allowed to stand for about half an hour, warming hastening the reaction. If nitrites be present from the reduction of the nitrate, a pink or red color develops, the control tube remaining colorless or perhaps slightly pink if it has absorbed a trace of nitrite from the atmosphere. If no nitrites are thus found, no reduction may have taken place, the nitrate remaining unchanged in the medium; or the reduction may have advanced beyond the formation of nitrite to the formation of nitrogen or ammonia. To determine these points the second inoculated tube is taken up. One-half its contents is poured into a clean test-tube and a strip of paper wet with Nessler's reagent suspended over the medium, when if fumes of ammoniacal character arise, the paper will slowly assume a yellowish or brownish-red color. The remaining half of the second inoculated medium is evaporated to dryness either in the tube or in a porcelain dish, and to the residue is added a drop of phenol-sulphonic acid (conc. sulphuric acid, c. p., 74 cubic centimeters; water, 6 cubic centimeters; and pure carbolic acid, 12 grams), and water added to dilute the mixture to one or two cubic centimeters. This is then alkalized by addition of sodium hydroxide solution. A yellow color indicates the persistence of the nitrate in the medium.

LESSON VIII.

ISOLATION OF BACTERIA IN PURE CULTURES.

It will be fortunate if after inoculation of nutrient substances and culture of the inoculated media the organisms grown should prove from their gross and minute characteristics to be of a single type. This may be expected only in instances where the efforts of the operator have from the first been directed, in the collection of the infected material, to the acquisition of but a single species, or in culture to permit conditions favoring the development of but a single type of organism; in the best directed attempts it is not uncommon, and in ordinary investigation of much of the material likely to be submitted for analysis it is invariable that there should occur several, perhaps a great variety of growths upon the media. It may naturally be inferred as impossible to study with any success the characteristics outlined in the preceding two chapters for any bacterium so long as it is confused with others. Hence arises in all bacteriologic study, whether of pathogenic or non-pathogenic forms, the necessity of Koch's second postulate: "*The organism to be studied must be obtained in pure culture.*"

The methods available for the isolation of organisms in pure culture arrange themselves readily into two groups: (a) *mechanical procedures*, and (b) *methods based upon physiologic peculiarities of the bacterium sought*.

(A) MECHANICAL METHODS.

1. **Plating Methods.**—When it is known from previous experience or may be reasonably inferred that a given material to be submitted to bacteriologic analysis contains a number of different organisms the exact nature of which is not known (for which reason it is impracticable to select a special method for isolation), efforts to widely distribute the organisms, and the colonies resulting in culture from their development, over an extended area, offer no little probability of success, so that with the sterilized needle each growth may be picked up and transferred to a separate container of sterile medium where it may develop alone. Any of the three modes of plating may be employed,—plates, Petri dishes, or Esmarch's tubes,—selection of one or other depending mainly upon the need of care to prevent contamination, but also partly upon the medium of growth selected, the conditions of temperature and atmosphere to be used in the development of the plated culture, and upon the extent of surface believed essential for proper dissemination of the bacteria. One would not select the rolled tubes if a wide surface be desired; yet they offer the greatest chance of remaining free from atmospheric contamination and are the most easily manipulated in incubator and anaerobic conditions. Plates and dishes are much more easily made if gelatine, rather than agar, be the medium used. Petri dishes are less liable to air contamination than plates, and are more conveniently placed in the incubator or anaerobic jar and more readily examined in the course of the culture. In any com-



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prehensive work it should not be neglected to arrange not one, but several plated cultures of the same material upon agar as well as upon gelatine, with a view of placing one culture at incubator temperature, one at room temperature, and a third in anaerobic surroundings. Each should be prepared from as nearly equivalent original material as possible, both in quantity and in kind, and the infected material thoroughly diffused through the liquefied medium before plating. (One must not forget to have the liquefied medium of as low a temperature as possible, lest harm be done to the bacteria diffused in it; and after diffusion, when about to pour the liquid upon a plate or in a dish, let it be kept in mind to flame and cool the lip of the tube for fear of contaminating the medium passing over it.) In addition to these agar and gelatine preparations, smears are to be made upon solidified blood-serum and submitted to incubator and room temperatures.

After proper growth has been attained and one can distinguish one type of colony from another, the sterile needle is to be used for transferring some of the organisms from each to separate tubes of sterile medium, identical with that used in the plated culture. Care is to be exercised in this step that the tip alone of the needle is touched only to that colony from which transfer is to be made, and that all other colonies in the preparation are avoided. It is not essential to have visible fragments or the entire colony on the needle. It will be found of advantage, before attempting the procedure, to bend the tip of the needle at right angles to its length, this shape lending itself to the operation. After the needle with the adhering organisms is withdrawn from the culture, it is introduced to the tube of fresh medium and a stroke or stab inoculation made, after which the needle is to be at once flamed. Each tube thus inoculated from the preliminary culture is for a time subjected to the same conditions as those which proved successful for the plated culture, and after sufficient development of the pure cultures the colonies in each are noted in the study of the gross and minute features of the organisms composing them.

The procedure is difficult only when the colonies to be separated are very close to each other in the plated culture; and care in manipulation alone will then insure success. Should one of the tubes inoculated prove to be mixed, the measure is to be repeated until successful.

Exercise 71.—Several loopfuls of *Bacillus prodigiosus* and *Micrococcus pyogenes* are mixed in a test-tube containing a few cubic centimeters of sterile water. Let each student diffuse a loopful of the fluid in liquefied gelatine and plate in one or other manner, subsequently producing from the plate a pure culture of each organism for inspection.

2. Salomonsen's Capillary Tubes.—Salomonsen suggested that after diffusion of the infected material has been effected in a sterile liquefied medium, a small amount of the latter be drawn into a sterile capillary glass tube instead of being spread out over a surface. The principle is, of course, the same as in any of the plating methods, the germs being disseminated, however, in a linear manner instead of over a plane. After the tube has been filled with the inoculated medium, the ends may be sealed in the flame or closed by wrapping a little sterilized cotton or paper closely about them. It must be realized that in such tubes, even if closed in the latter manner, but little air access is permitted to the organisms in the medium any distance from the ends; and as a matter of fact it is especially in the isolation of anaerobic varieties

(facultative and obligate) that the method is of value. It is the practice of the writer to prepare a number of capillary tubes, inclosing them in a large glass tube like that used in Hesse's air apparatus, both ends of which are filled with cotton plugs. In this they are sterilized in the oven and kept until used. When filled with an inoculated medium and closed, each capillary is marked with the wax pencil so that it may be surely distinguished and placed for culture in a second sterile glass tube like the first in order to prevent the outside surface of the capillary from contamination and to allow observation during culture. It will be found convenient to attach to this containing tube a strip of card or paper on which may be marked the time and appearance of each colony for each of the contained capillaries as it appears, as is indicated in the accompanying diagram (Fig. 67). Several capillaries may be kept in the enveloping tube if properly marked to prevent confusion. When growth appears in such a capillary at any distance from the ends, whether the ends have been sealed or not, it may be accepted as either a facultative or obligate anaerobic colony; when the tube is sealed, all growth appearing is anaerobic; should the ends not be sealed and growth occur close to the end, it may, however, be an aerobic variety.

When it is desired to transfer the colonies to separate culture tubes, a capillary is withdrawn from the large container and with a sterile forceps a short length bearing

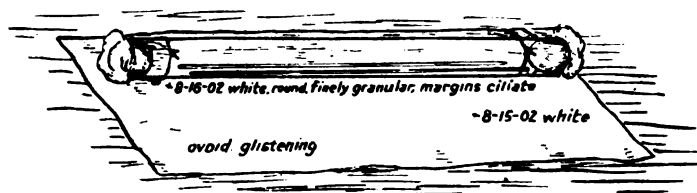


FIG. 67.—SALOMONSEN'S CAPILLARY TUBES INCLOSED IN PROTECTIVE GLASS TUBE, WITH LATTER ATTACHED TO CARD ON WHICH ARE PRESERVED NOTES OF COLONIES WITHIN.

the colony broken out (one end of the fragment should be very close to the colony) and dropped into sterile medium. (It is well first to transfer to bouillon into which diffusion from the colony in the capillary fragment readily takes place; after growth in the bouillon one or two loopfuls are smeared over the surface, or a stab made with the straight needle into the interior of fresh solid medium.) The medium inoculated with the colony from the fragment of the capillary tube should for several days be grown in the anaerobic jar, its growths transplanted, and the medium then exposed for some days to the ordinary atmosphere in order to afford a chance of development to any aerobic individuals which possibly may be present.

This method is especially valuable in isolation of the anaerobes, facultative or obligate, in studies of blood or other pathologic material taken from some diseased individual, such a fluid being drawn directly into the capillary tube and sealed therein, where it serves as the culture medium for the preliminary growth (McLaughlin).

Exercise 72.—A tube of liquefied gelatine is inoculated with a loopful each of a bouillon culture of *Bacillus typhosus* and *Pseudomonas pyocyanea*. Let each student recover in pure culture and present for inspection the former of these organisms by the above method.

3. **Kleb's Fractional or Dilution Method.**—This is a modification of the ordinary plating methods introduced for use in case the original material is believed to contain a large number of organisms, and where it is feared the degree of bacterial presence is so great that a plate made from the medium inoculated with the undiluted substance will be hopelessly crowded with colonies. It is essentially the same method of fractional or dilution inoculation described in connection with the instruction for the counting of bacteria in any material to be analyzed (page 162). It is very popular, being perhaps more than any of the other mechanical methods employed in isolation work. In this practice it is hoped by serial dilutions of the original material to diminish the number and thoroughly disseminate the different types of bacteria present so as to obtain in a final inoculation growth of but a single variety or at best of but a few types. These dilutions of the infected material may be made either in sterile water before inoculation, or by diffusions or smears in the medium of growth in the course of inoculation. The latter is the method ordinarily employed. For example, in the isolation of the mycobacterium of diphtheria from the material obtained from the throat of a patient, a tube of liquefied blood-serum is first inoculated by rubbing the swab on which the exudate has been collected upon the surface of the medium. The swab is now returned to its protective tube and the platinum loop taken up. This, having been sterilized in the flame and cooled, is drawn over the surface of the serum in this first tube and then carried over the surface of sterile serum in a second tube. It is again flamed and cooled and drawn over the inoculated serum of the second tube, and the infection adhering to it carried to sterile serum in a third tube. It is to be supposed that in these transferences but a small number of the original organisms have found their way into the third tube, and these few are probably widely scattered over the surface of the serum. Each tube is marked in proper manner and placed in the incubator. The first growths to appear in the medium, usually within eighteen or twenty hours, are the small white, punctate colonies of *Mycobacterium diphtheriae*. They are most definite and easily recognized in the third tube; and from this, as soon as discovered, should be transferred with the needle to a fresh tube of serum, a stroke inoculation being made. (A film is also to be prepared and stained with Loeffler's blue for confirmation.) Later, in all three tubes, especially in the first and second, appear in greater or less profusion the colonies of the pus germs and other organisms, often to utter confusion.

The same principle is followed where the dilution is made by diffusion in liquefied gelatine or agar. A small quantity of the infected matter, as contaminated water, is planted by diffusion in a tube of liquefied gelatine. From this tube a small quantity (a loopful, or a definitely measured amount, representing a certain proportion of the original quantity of the fluid examined, if study of the number of bacteria is also to be pursued) is transferred to a second tube of liquefied gelatine and diffused; from this a similar quantity is transferred to a third tube and likewise diffused in the liquefied medium in it. All three tubes are plated in one or other manner, and the plated cultures placed in proper surroundings for development. In the last, from the diminished numbers of germs present, the colonies will probably be well separated from each other, and transfer by means of the needle to fresh medium possible. The first and perhaps the second cultures will probably be too crowded with growth to permit advantageous manipulation, but they will at least serve as controls to establish whether in the third culture all the types of bacteria in the original material have been obtained.

Exercise 73.—Let each student make fractional smears from material obtained from the fauces of a diphtheritic patient; or if this be not at hand, let the same be done with a mixture of known diphtheritic organisms and some of the pus cocci diffused in a tube of bouillon.

Exercise 74.—Let each student isolate the organisms in a loopful of normal saliva by diffusion in liquefied gelatine and agar.

(B) PHYSIOLOGIC METHODS.

In this group of procedures advantage is taken of some peculiarity in the life-history of an organism sought to be obtained in isolation, either its peculiar resistance to some influence capable of destroying associated bacteria before inoculation, or some method of culture capable of restraining the development of organisms present with it in the inoculated medium. These physiologic methods are selective, and for the most part are employed where some definite organism is sought, and are not applicable for the isolation of every species of any complex mixture.

1. *Selection by Living Animal Tissues.*—Advantage may be taken of the fact that the living tissues of different animals by nature afford to some organisms a favorable opportunity for growth, while to others they are resistive in variable degree, perhaps to the extent of actual destruction of the germs. It is to this fact largely that we refer the distribution of disease in different varieties of animals. If a complex mixture of bacteria in which there exists some form especially pathogenic for a certain kind of animal be inoculated into an individual of this species, it is to be presumed that the pathogenic germs will, from the favor of the conditions afforded, undergo rapid development, probably showing a specific selection for some particular structure of the animal body; while it is probable that the other organisms, finding less favorable surroundings, will either not develop at all or but poorly (and in the latter case be restricted to the immediate vicinity of the point of inoculation). It then is possible, by planting upon proper laboratory media from the parts of the animal which have been especially invaded by the pathogenic organisms, to obtain them free from their former associates. If, for example, one will inoculate beneath the skin of a male guinea-pig some of the nasal discharge from a horse affected by glanders, containing a mixture of *Bacterium mallei* with various pathogenic and perhaps other organisms, the former rapidly invade the animal, selecting the testicles as special points of growth, and shortly kill it; while the pus germs are apt to remain restricted to the subcutaneous tissues about the point of injection. By planting some of the material obtained from the more or less necrotic testicles upon the laboratory media a pure culture of the glanders organism may without special difficulty be obtained.

Exercise 75.—Early in the work of the class some sputum from a consumptive case should have been dissolved in physiologic salt solution and injected with a hypodermic syringe into the subcutaneous tissues of the abdomen or chest of a number of guinea-pigs. In some of the animals it is probable that the suppurative changes caused by the pyogenic germs in the sputum may destroy life at a comparatively early period, but it may be expected that in other instances the animals will in the course of some days have overcome any general or local influences of these bacteria. In such,

at a later period, usually about three or four weeks, the evidences of tubercular disease will be manifested by the appearances of enlarged and caseated lymphatic glands and by emaciation of the animals. Such an animal is killed and scrapings planted with the usual precautions upon solidified blood-serum (select fresh, undried medium), the substance being well rubbed into the surface of the serum, and the inoculated tubes placed at body temperature in the incubator. In ten or twelve days pure growths of *Mycobacterium tuberculosis* should be obtained, and may be identified by their gross and minute characteristics as set forth in any of the various descriptive works on bacteriology.

9 **2. Selection by Culture Medium.**—In a like manner the different nutrient media, because of peculiarity of their organic matter or the presence of restrictive or favoring inorganic constituents, may exhibit a special fitness for the nourishment of some particular germ, to the more or less absolute exclusion of associated microbes and thus lead to the early and preponderating development of the former, while the latter are inhibited from growth in the culture either entirely or in part. This may be noticed in the rapid development of the diphtheritic organism on blood-serum, Loeffler's medium, or ascitic agar (agar made up with ascitic fluid instead of bouillon, and with five per cent. of glycerine added), the colonies of this microorganism appearing a number of hours earlier on such medium than those of the commonly associated pus bacteria; while on ordinary agar or gelatine this is almost or quite reversed.

For this reason successful search for some definite form of bacterium demands thoughtful selection of the media to be employed for its culture. It may not be, however, that the material selected will favor isolation by increase of the rate of development of the organism sought for, or by exclusion of growth of its associates; all may grow with equal rate, but the medium may have caused the desired germ to take on some well-marked gross cultural peculiarities, so that its colonies are easily recognized from the rest and their selection for transfer to fresh medium made certain.

In this connection may be mentioned the practice of adding *special substances to the nutrient medium* for the purpose of inhibiting the growth of undesired bacteria in the culture, the substances thus used having little or no influence upon the development of the particular microorganism which it is desired to obtain in pure culture. Carbolic acid, hydrochloric acid, iodide of potassium, and other substances are sometimes added, with this in view, to the nutrient upon which fecal matter is implanted for the purpose of restraining the growth of organisms other than *Bacillus coli* or *Bacillus typhosus*; the employment of such substances also bringing out differences in the rate of development and of gross cultural appearance as will permit the recognition of these two organisms from each other in the culture, and consequently their mechanical separation.

Exercise 76.—With the sterile needle make stroke or smear inoculation of normal human feces upon a dish of Elsner's medium and grow for twenty-four hours (if the medium be made up with agar instead of gelatine, one may place the inoculated dish in the incubator). At the end of this time distinct colonies of a pale brownish tint may be recognized as the growth of

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Bacillus coli, the other bacteria of the fecal matter having probably entirely failed of development. Plant from such colonies to other sterile media for comparison with the known characteristics of *Bacillus coli*.

Exercise 77.—From a known mixture of typhoid and colon bacilli plant in the same manner upon the same medium. Note at the close of twenty-four hours the appearance of the colonies of the colon bacillus. After a second day the colonies of *Bacillus typhosus* appear like tiny, clear, colorless droplets. Separate to fresh media (agar, gelatine, and potato) and study for each organism the macroscopic cultural features, and the flagellation, staining by Gram's method, liquefaction of gelatine, gas formation in sugar-containing bouillon, acid production, milk coagulation, indol production, and the agglutination phenomenon on addition of serum from a suitable typhoid subject.

3. Selection by Culture Temperature.—From reflection upon the differences of optimum temperature for growth of the different groups it may be readily appreciated that this feature may be utilized in efforts to separate from complex mixtures the psychrophilic, mesophilic, or thermophilic bacteria (*vide* exercise 35). It need scarcely be added that, having provided the condition of temperature requisite for growth of some bacterium which it is desired to separate from such a mixture, the first colonies appearing in the inoculated medium are, other things being equal, those of the sought-for organism. These are at once to be transferred to fresh medium for inquiry as to perfect isolation and confirmation of type, lest if allowed a longer period of development other germs may follow if the temperature to which the culture has been subjected be within the vital range of such forms.

4. Selection by Culture Atmosphere.—Similarly, the possibility of growth in the ordinary air or in anaerobic surroundings may be utilized in the separation of the obligate forms of the two varieties of bacteria, it being a matter of no great difficulty to isolate and obtain in pure culture one or other variety where only the obligate forms are present in a mixture. The facultative forms may, however, grow along with the aerobes in ordinary air or along with the obligate anaerobes in atmospheres from which oxygen has been displaced by hydrogen or nitrogen. In such cases it may be that a difference in the rate of development will serve to render possible the distinction of obligates from the facultative varieties, or a proper selection of temperature or medium of growth in connection with atmosphere may lead to the same results. The capillary tubes of Salomonsen, or Koch's suggestion that on the surface of a plated medium in ordinary air a small sterile plate of glass or mica be applied to render the underlying medium relatively anaerobic, may be utilized in this relation; but commonly the anaerobic jar with special interior atmosphere or the special anaerobic tubes described in a previous lesson are employed in such work.

Exercise 78.—Make an infusion of ordinary garden earth in sterile water and from this make smear inoculations on agar surfaces. Grow in an atmosphere of hydrogen at body temperature. Observe after twenty-four hours the appearance of small, whitish, round colonies with delicate veil-like margins. Transfer by stab inoculation to gelatine and agar in at-

mospheres of hydrogen. After identification of the gross characteristics as those of *Bacillus tetani*, study the minute appearances, sporulation, flagellation, staining by Gram's method, coagulation of milk, acid production, gas production, and indol production for comparison with the known properties of this organism. (Note.—It is by no means necessary that *Bacillus tetani* be the only anaerobe likely to be obtained from such source; it is, however, a common one, and others which may be obtained in mixture or alone are to be carefully differentiated from it.)

5. Cohn's Heating Method.—This procedure is intended for the separation of spore-forming bacteria from vegetative bacteria, and depends upon the well-known resistance of spores to temperatures fatal to ordinary germs. An infusion known to contain spores of some organism, as well as various vegetative varieties, is boiled for one minute (or, when it is known that the spores are very resistive, for a longer time), by which means all the adult types of bacteria are usually destroyed, most, if not all, of the spores being uninjured by the heat. It is now quickly cooled and from it inoculations are made in the usual manner in the usual media and the cultures placed in proper surroundings for development of the spores (media best suited for germination of the spores are those containing plenty of moisture, as bouillon). There may, of course, be spores of several bacterial varieties present in the material, and in such case the colonies resulting from development of the spores are to be separated from each other by other suitable methods.

Exercise 79.—Make an infusion of hay by placing a bunch of hay in a jar of water and allowing it to soak over night at incubator temperature. The following morning the reddish infusion is strained from the hay through a piece of cheese-cloth and boiled for one minute. Cool by placing the container in a vessel of cool water. Plant from the boiled fluid to a number of tubes of agar, gelatine, and bouillon, and allow to grow in the ordinary atmosphere at room temperature. In a day or two the characteristic appearances of the hay bacillus will appear without contamination with the other organisms which were probably present upon the hay.

Or, having strained the infusion from the hay, dilute it with water until it is about 1006 in specific gravity and neutralize by the addition of sodium hydrate (litmus used as indicator). Distribute the fluid in a number of sterile tubes, boil for one minute, and then place in the incubator or in room temperature. In a day or two a dense white surface growth of *Bacillus subtilis* will have been obtained.



LESSON IX.

CLASSIFICATION AND IDENTIFICATION OF BACTERIA.

No thoroughly satisfactory classification of the bacteria has as yet been proposed and will probably be wanting until our knowledge of their characteristics is sufficiently developed to render certain their true generic relations. The older classifications, dealing with the so-called oligomorphous varieties, were based entirely upon the shape of the individual bacterial cells, a system continued to the present by a number of writers; prominent functional characteristics, as the power to liquefy gelatine, serving in a measure to subdivide the major groups. It is not to be supposed that there does not exist sufficient natural basis for arrangement of these forms of life into orders, families, genera, and species, as is done in case of the higher vegetables and animals; but it must be acknowledged that from the insufficiency of our methods of study there is not as yet that amount of information which will permit a stable arrangement such as is established in the studies of higher botany and zoology. Whatever classification is adopted must for the present, therefore, be regarded as tentative. Probably the best classification for working purposes thus far suggested is that proposed by Migula (*System der Bakterien*, 1900); and this, with certain emendations by Chester (*Determinative Bacteriology*, 1901), and further minor modifications approaching more closely to the arrangement of Migula, is adopted in these pages. It is unfortunate that its generic system is based upon a structural feature of bacteria which is difficult of demonstration and apparently not invariable throughout the life-history of the individual cells or under all conditions—viz., the presence, number, and position of flagella. It is possible, however, that in the future much of this objection may be relieved by the discovery of more reliable methods of demonstrating these flagellar appendages than those now known, when perhaps it will be found that these organs are not so variable as would to-day appear. Or it is possible that with the advance of our knowledge of the chemistry of bacterial constitution some more constant feature may be found upon which to base a classification of convenient genera. Until the acquirement of some such desiderata, however, the writer is disposed to regard such an arrangement as that of Migula with much appreciation, as of no little use in reducing to system a study which has been marked by much confusion of excellent as well as careless work.

Recognizing the entire group of the schizomycetes or fission fungi as an order, the classification adopted would divide it into two suborders, the *Eubacteria* (corresponding roughly with the oligomorphous bacteria of various writers), the individual cells of the members of which have no granules of sulphur or bacteriopurpurin in their constitution; and the *Thiobacteria* (roughly, the pleomorphous bacteria of writers), in the cells of which are to be seen red or violet granules of sulphur or bacteriopurpurin.

The eubacteria or true bacteria are divided into five families from morphologic peculiarity of the cells: (I) *Coccaceæ*, spherical forms; (II) *Bacteriaceæ*, straight, rod-

shaped, unbranched forms without definite envelope; (III) *Spirillaceæ*, curved or spiral rods, unbranched, without envelope; (IV) *Mycobacteriaceæ*, rod forms, often with irregularly clubbed ends, sometimes forming filaments, showing some individuals with true branching, usually straight, but sometimes slightly curved; and (V) *Chlamydobacteriaceæ*, filamentous bacteria composed of rod-shaped cells and surrounded by a distinct sheath, without granules in the cell contents.

The thiobacteria are divided by Migula into two families: (I) *Beggiatoaceæ*, filamentous bacteria containing sulphur granules in the cell contents; and (II) *Rhodobacteriaceæ*, non-filamentous, with bacteriopurpurin or sulphur granules, red or violet in color, in the cell contents. The latter family is further divided into five subfamilies: (A) *Thiocapsaceæ*, cell division in three planes; (B) *Lamprocystaceæ*, cell division first in three, then in two planes; (C) *Thiopedaceæ*, cell division in two planes; (D) *Ameobacteriaceæ*, cell division in one plane; (E) *Chromateaceæ*.

The following synopsis of the order indicates the relations of the suborders, families, and genera:

Order: SCHIZOMYCETES.

- (A) Suborder: **EUBACTERIACEÆ** (without colored granules in cell contents; uncolored except in a very few species, then faintly, generally green).
 - I. Family: **Coccaceæ** (globular, becoming slightly elongated before cell division; cell division in one, two, or three directions).
 - (a) Genus: *Streptococcus* (cell division in one direction, united in chains, non-flagellated).
 - (b) Genus: *Micrococcus* (cell division in one, two, or three directions with separation of cells; non-flagellated).
 - (c) Genus: *Sarcina* (cell division in three directions, united in packets of eight; non-flagellated).
 - (d) Genus: *Planococcus* (cell division in one, two, or three directions, cells separate; flagellated).
 - (e) Genus: *Planosarcina* (cell division in three directions, cells united in packets of eight; flagellated).
 - II. Family: **Bacteriaceæ** (cells straight, cylindric, short, oval to rods and filaments; without sheath; no true branching; with or without flagella).
 - (a) Genus: *Bacterium* (cells straight, cylindric, oval to rods or filaments; non-motile, without flagella; endospores present or absent).
 - (b) Genus: *Bacillus* (cells straight, cylindric, oval to rods or filaments; motile, with flagella varying in number, peritrichous; endospores present or absent).
 - (c) Genus: *Pseudomonas* (cells straight, cylindric, occasionally in short filaments; motile, flagella monotrichous or amphitrichous; endospores known in only a few species).
 - III. Family: **Spirillaceæ** (cells more or less curved; cell division transverse to long axis of cells; usually without endospores; with or without flagella; flagella few, monotrichous or amphitrichous).
 - (a) Genus: *Spirosoma* (cells rigid, without flagella).
 - (b) Genus: *Microspira* (cells rigid; one, rarely two or three polar flagella).
 - (c) Genus: *Spirillum* (cells rigid; a bundle of polar flagella).
 - (d) Genus: *Spirochata* (cells flexile, sinuous).



- IV. Family: **Mycobacteriaceæ** (cells straight, short or long, cylindric, clavate, cuneate in form; at times showing a true branching, or as long, branched mycelial filaments; no sheath; without endospores, but with formation of gonidia-like bodies due to transverse segmentation of cells).
- (a) Genus: *Mycobacterium* (cells commonly short, cylindric rods, sometimes bent and irregularly swollen, clavate or cuneate; may show Y-shaped forms or longer filaments with true branchings; may produce short coccoid elements which are perhaps gonidia).
 - (b) Genus: *Streptothrix* (cells commonly long-branched filaments; produce gonidia-like bodies; form aerial hyphæ in cultures, causing resemblance to moulds).
- V. Family: **Chlamidobacteriaceæ** (filaments composed of rod-shaped cells, and surrounded by a distinct sheath; cell division transverse or in three directions, resulting in formation of gonidia-like bodies which may or may not be motile).
- (a) Genus: *Leptothrix* (filaments unbranched; division transverse).
 - (b) Genus: *Phragmidiothrix* (filaments unbranched; divisions in three directions; sheath scarcely visible).
 - (c) Genus: *Crenothrix* (filaments unbranched; division in three directions; sheath distinct).
 - (d) Genus: *Cladothrix* (filaments show false branching).
- (B) Suborder: **THIOBACTERIACEÆ** (cells show presence of colored granules, or sometimes diffuse coloring, red or violet).
- I. Family: **Beggiatoaceæ** (filamentous; with or without sheath; motile or non-motile; sulphur granules in cell contents; gonidia formation not known).
- (a) Genus: *Beggiatoa* (filaments motile by means of undulating membrane; segmentation not apparent except when stained with iodine; colorless or faintly reddish-violet).
 - (b) Genus: *Thiothrix* (filaments non-motile; surrounded by delicate sheath; sulphur granules in cell contents; at ends of filaments rod-shaped gonidia; filaments unequal in diameter).
- II. Family: **Rhodobacteriaceæ** (cells irregular, globular, oval, cylindric, non-filamentous; contents show the presence of sulphur granules or bacterio-purpurin, red or violet).
- (A) Subfamily: **THIOCAPSACEÆ** (cells divide in three planes).
 - (a) Genus: *Thiocystis*.
 - (b) Genus: *Thiocapsa*.
 - (c) Genus: *Thiosarcina*.
 - (B) Subfamily: **LAMPROCYSTACEÆ** (cells divide in three planes, then in two).
 - (a) Genus: *Lamprocystis*.
 - (C) Subfamily: **THIOPEDEACEÆ** (cells divide in two planes).
 - (a) Genus: *Thiopedia*.
 - (D) Subfamily: **AMEBOBACTERIACEÆ** (cells divide in one plane).
 - (a) Genus: *Amebabacter*.
 - (b) Genus: *Thiothece*.
 - (c) Genus: *Thiodictyon*.
 - (d) Genus: *Thioplyococcus*.

(E) Subfamily: CHROMATIACEÆ.

(a) Genus: *Chromatium*.(b) Genus: *Rhabdochromatium*.(c) Genus: *Thiospirillum*.

In the study of any unknown bacterium the student should have in his possession all the data which are obtained in a systematic completion of some blank form as provided in the Appendix of this volume. Furnished with this information, the identification may be attempted by reference to the analytic keys in various systematic works on bacteriology (Chester, Migula, Neumann and Lehmann, Sternberg, Flüge, Hueppe, Fischer, Macé, *et al.*). In case of typical forms the recognition by such reference is not a matter of great difficulty; but, unfortunately, owing both to the insufficiency of our knowledge arising from inefficiency of methods of demonstration, faults of description of many species, and consequent confusion of system, and to the greater or less variability apt to be met in a given species, difficulties do arise for the experienced as well as the inexperienced. Patience and care of observation are absolute essentials. As far as they go, our methods, if properly applied, are reliable, and when in difficulty the student should recall the possibility of bacterial variation as giving rise to his trouble and extend his observations to other examples of the species under investigation, in hope of detecting the source of apparent discrepancy. Methodical, painstaking effort will always yield some measure of success; and if certain recognition of the species is impossible to the student, at least it will be possible to refer temporarily and tentatively the bacterium studied to some class typified by some well-known organism, after which the subsequent work is narrowed to differentiation from the other members of the same class.

The scope of this work scarcely permits the publication of any extended analytic key, for which reference to the descriptive texts must be made; however, for convenience in the preliminary classification, the following outline, based upon the above table of classification, and adapted with some changes from Chester's *Determinative Bacteriology*, is presented, leading to the separation into genera of the true bacteria. Thereafter follows a list of equivalents of the generic names to which reference may be made in the use of reference works other than Chester in the determination of species.

- (A) Cell contents devoid of bacteriopurpurin or sulphur granules, red or violet. (Chester recognizes among this group a few forms as thiospirillum, thiothrix, and a class of pseudomonads which contain sulphur granules, which in the above table of classification are, however, placed among the thiobacteriaceæ),.....EUBACTERIACEÆ.
- I. Cells globular, free or united, usually non-motile, mostly without flagella,.....Family: COCCACEÆ.
- (a) Cells without flagella; division in one plane (cells united in one direction),.....Genus: *Streptococcus*.
- (b) Cells without flagella; division in one, two, or three planes to formation of individuals, merismopodia or zooglea masses,.....Genus: *Micrococcus*.



- (c) Cells without flagella; division in three planes to form packets of eight or geometric multiples, Genus: *Sarcina*.
- (d) Cells with flagella; division into separate individuals, Genus: *Planococcus*.
- (e) Cells with flagella; arranged in sarcina packets, Genus: *Planosarcina*.
- II. Cells short or long, straight, cylindric; separate or in chains; without sheath surrounding chains; with or without endospores; motile or non-motile; with or without flagella, Family: **Bacteriaceæ**.
 - (a) Cells without flagella; endospores present or absent, Genus: *Bacterium*.
 - (b) Cells with flagella; peritrichous; endospores present or absent, Genus: *Bacillus*.
 - (c) Cells with flagella, polar; endospores usually absent, Genus: *Pseudomonas*.
- III. Cells more or less spirally curved; division transverse to long axis of cells; usually without endospores; with or without flagella, usually polar, Family: **Spirillaceæ**.
 - (a) Cells rigid, comma-shaped to spiral; non-motile, without flagella; without known endospores; cells single or in zooglea, Genus: *Spirosoma*.
 - (b) Cells rigid, usually weakly curved comma-shaped or short spirals; each cell with one (rarely two or three) polar flagellum; endospores unknown, Genus: *Microspira*.
 - (c) Cells rigid, comma-shaped or spiral, of variable thickness; cells actively motile with a bunch of four or more flagella at one or both poles; a few forms with endospores, Genus: *Spirillum*.
 - (d) Long, slender filaments; cells flexible; undulatory or snake-like movement not progressive or about the long axis; flagella not known; endospores apparently absent, Genus: *Spirochæta*.
- IV. Cells either short or long, cylindric-clavate-cuneate in form, sometimes showing true branching, or as long, branched mycelial-like filaments; filaments not surrounded by a sheath; without endospores, but with formation of gonidia-like bodies by transverse segmentation of rods or filaments, Family: **Mycobacteriaceæ**.
 - (a) Cells ordinarily short cylindric rods, often bent, irregularly swollen, clavate or cuneate, sometimes in Y-shaped forms or in longer filaments with true branchings; without flagella; without endospores, but may present short coccoid elements, perhaps gonidia, Genus: *Mycobacterium*.



- (b) Cells ordinarily long-branched filaments; non-motile; without endospores, but produce short gonidia-like bodies by multiple segmentation of filament; cultures on solid media raised, dry, rough, crumpled, often mould-like from formation of aërial hyphæ,.....Genus: *Streptothrix*.
- V. Filamentous bacteria, composed of rod-shaped cells; filaments surrounded by sheath; cell division into motile or non-motile gonidia,..Family: **Chlamidobacteriaceæ**.
- (a) Filaments unbranched; non-motile; inclosed in delicate or thick sheaths; fixed or in slimy masses. Cell contents become segmented into round or oval gonidia by transverse division; gonidia non-motile. (Separation of filaments and presence of sheath shown by iodine staining),Genus: *Leptothrix*.
- (b) Filaments unbranched; surrounded by very delicate sheath seen only in old filaments; filaments consist at first of rods in one plane, which later divide in three planes to form sarcina-like packets; later the cells become spherical and free,.....Genus: *Phragmidiothrix*.
- (c) Filaments unbranched, surrounded by definite sheath, fixed, usually thinner at base than at free end; cell division in three directions to formation of gonidia (*micro-* and *macro-gonidia*), which may escape or germinate in filament,Genus: *Crenothrix*.
- (d) Filaments show false branching, due to intercalary growth of a cell through the sheath laterally; filaments generally with delicate sheaths, often fixed and forming tufts; reproduction by formation of motile gonidia (swarm spores) which bear a bundle of flagella laterally to one pole,.....Genus: *Cladothrix*.

LIST OF MORE OR LESS EQUIVALENT TERMS IN GENERIC NOMENCLATURE.

- Streptococcus*, Billroth (*diplococcus*; *leuconostoc*, Van Tieghem; *ascococcus*, Cienkowski).
Micrococcus, Hallier-Cohn (*coccus*; *sphaerococcus*; *tetrad*; *merismopedia*; *merista*; *pediococcus*; *staphylococcus*, Ogston; *leucocystis*, Schrötter; *hyalococcus*, Schrötter; *lampropedia*, Schrötter; *bacteridium*, Schrötter; *ascococcus*, Billroth; *monas*, Ehrenberg).
Sarcina, Goodsir.
Planococcus, Migula (*id. micrococcus*).
Planosarcina, Migula.
Bacterium, Ehrenberg-Migula (*bacillus*, Cohn; *bacteridium*, Davaine; Neumann and Lehmann and others reserve the name for rods devoid of endospores).

Bacillus, Cohn-Migula (*bacterium*, Hüppe; Neumann and Lehmann and others employ the term as indicating rods containing endospores; includes special forms, as *clostridium*; *paraplectrum*; *ascobacillus*; *proteus*, Hauser; *halibacteria*, etc., Fischer).

Pseudomonas, Migula (terms applied to last two genera; *monas*; includes the class *nitromonas* of Winogradsky).

Spirosoma, Migula (*comma-bacillus*; *spirillum*, Ehrenberg; *microspira*, Schrötter; *vibrio*, Müller).

Microspira, Migula (*spirillum*, Ehrenberg; *vibrio*, Müller; *comma-bacillus*).

Spirillum, Ehrenberg-Migula (*vibrio*, Müller; *comma-bacillus*).

Spirochæta, Ehrenberg.

Mycobacterium, Lehmann and Neumann (includes *corynebacterium*, Lehmann and Neumann; *sclerothrix*, Metschnikoff).

Streptothrix, Kruse (*actinomyces*, Harz; *cladothrix*, Günther; *oöspora*, Wallroth).

Leptothrix, Kützing (*streptothrix*, Migula).

Exercise 80.—Let the instructor, having placed upon the blackboard the characteristics of some known microorganism, require of each student the practice of identification of the bacterium from the data given, following some selected analytic key; repeat with the characteristics of other bacteria as desired.

LESSON X.

STUDY OF THE PATHOGENIC ACTION OF BACTERIA.

While all bacteria are necessarily of general interest as being either indirectly or directly important to our life and relations, those which produce disease in man are of essential interest to medical men. To these the name *pathogenic bacteria* is commonly applied, the remainder being termed *non-pathogenic bacteria*. The known pathogens form a comparatively small proportion of the numerous species thus far discovered; but it should not be forgotten that we have little idea of the possibility of pathogenic power which under even apparently unimportant modification of condition may reside in the so-called non-pathogens. Anthrax is ordinarily non-pathogenic to chickens, whose body temperature is comparatively high; but should the hen's body heat be lowered several degrees by refrigeration, she becomes susceptible to the influences of the bacterium of this disease. The relative amount of acid or alkaline substances which *Microspira comma* meets in the alimentary canal of man apparently determines whether he shall escape or be stricken with Asiatic cholera; and *Bacillus coli*, a constant and profuse parasite of man's intestinal canal, is often met in other situations in the body, apparently the cause of serious lesions. It is not impossible that every bacterium may somewhere in the range of higher life, animal or vegetable, find itself capable of a parasitic existence; such parasitism may doubtless be forced by transient and perhaps unobserved special circumstances or conditions. Parasitism does not necessarily imply pathogenesis; but should it happen that the host is susceptible to the various influences exerted by the parasite and its products, then disease must arise. It is from such a view not a wonderful thing that diseases unusual to man should occasionally appear, their entrance and development depending upon some accident or some temporary and unusual condition; nor can it be looked on as impossible that totally new disease occurrence may take place, depending upon a forced invasion of some microbe hitherto unknown as a human parasite and its development favored by some unusual condition which may exist in the body of the affected individual. Correlatively, while we are accustomed to speak of certain microbes as being *obligate parasites*, this is probably only in a general sense correct; in strict meaning it can scarcely be doubted that under unknown but definite conditions the parasites of man might be reduced to parasitism in some other species or even to a saprophytic existence. Thus is explained the fact that human disease of some sort may sometimes apparently die out and be unknown for a greater or less period, returning, when conditions favor, to the surprise and consternation of men.

It is well, therefore, to think of all bacteria as having a possible pathogenic influence sometimes and to some species of created beings; and no matter what the source of the microorganism studied, a part of that study forced upon us for our own protection must concern itself with the influence the bacterium may exert upon higher life. It is impossible, of course, that its relations to all forms of life be investigated;

as a rule we are satisfied with experimentation upon one or two species of the smaller animals easily available for laboratory work, as frogs, hens, pigeons, mice, rats, guinea-pigs, rabbits, and dogs. This part of the study is dictated in the third of Koch's postulates relating to the study of pathogens and the proof of their causal relationship to disease: "*The bacterium in pure culture is to be inoculated into a susceptible animal and its effects compared with and recognized as similar or identical to the known symptoms of the originally diseased individual.*"

The selection of animals susceptible to the influence of an organism is by no means always easy or possible in the small range available to the laboratory worker. In general, however, it may be said that frogs are well suited for the psychrophilic bacteria, and the small warm-blooded animals for the mesophilic varieties. After inoculation the animals are to be kept in as nearly normal relations and surroundings as possible, and it is well always to place in the same conditions, but in separate containers, one or more similar but uninoculated animals as controls. The inoculations may be made in one or other of a variety of ways. Sometimes a mere scratching of the skin, as in vaccination, with the culture laid on the surface and well rubbed in with a small spatula, is sufficient. Or a bit of thread moistened with a fluid culture may be drawn through the skin after the manner of applying a seton. Usually, however, more definite operations are required, and are preferable from more or less certainly avoiding the entrance of the common bacteria of the skin as contaminating and confusing influences, hypodermic, intravenous, and intraperitoneal inoculations being the usual forms in the warm-blooded animals.

Hypodermic Inoculations.—Mice are best held by the end of the tail in a glass jar, the tail being drawn out over the edge of the jar and the mouse held in the jar by a board over the top of the latter so placed as to permit the exposure of the tissues at the base of the tail only. Guinea-pigs and rabbits may be held by an assistant, the two hind legs in one hand, the two fore legs in the other; or one of the various mechanical animal holders (Fig. 68) may be employed. Dogs had best be fastened for any important inoculation in one of the troughs commonly used for this purpose in experimental work, although for inoculation with the syringe this is scarcely necessary. Pigeons and hens should be held with one hand grasping the legs, tips of the wings, and the tail, and the other holding the neck in extension. Mice and rats are usually inoculated in the tissues about the base of the tail and along the lower end of the spine; rabbits and guinea-pigs, in the tissues of one side of the thorax; pigeons and hens, in the pectoral region; the culture being usually introduced into one of the subcutaneous lymph-sacs when frogs are used as experiment animals.

It is next to impossible to thoroughly sterilize the skin, but the hair or fur should be removed by clippers and razor or the feathers plucked from a small area about the site of the proposed inoculation and the surface well washed with soap and water and rinsed with boiled water (or a pledget of cotton soaked with a 1:1000 solution of corrosive sublimate applied closely to the surface for a few minutes, alcohol and ether being afterward used for rinsing). The culture may be suspended in sterile water or physiologic salt solution, or an active bouillon culture employed, when it is best introduced beneath the skin by means of a syringe. Or a dry culture may be inserted into a small pocket under the skin upon a stiff platinum needle. The hypodermic syringe used should be so constructed that it may be thoroughly sterilized, made of glass, metal, and rubber (as Koch's or Strohschein's). An excellent substitute may be extemporized from a glass pipette or Sternberg's bulb with a hypodermic needle attached to the tube by means of a rubber tube, the fluid being forced from

the pipette by blowing into the upper end, which is plugged with a sterile cotton stopper, and from the bulb by the application of heat. The syringe and all its parts should have been sterilized by boiling or in the autoclave, or by prolonged immersion in a disinfecting solution, with subsequent rinsing in sterile water. The fluid in which the bacteria are suspended having been drawn into the syringe, a fold of the skin is pinched up with the fingers or a pair of forceps and the needle is thrust into the subcutaneous tissues of the fold, penetrating some distance from the point of entrance. The fluid is then forced gently and steadily into the tissues and the needle then withdrawn. It is usually unnecessary to do anything more; but if desired, a drop of collodion may be applied to the point of puncture.

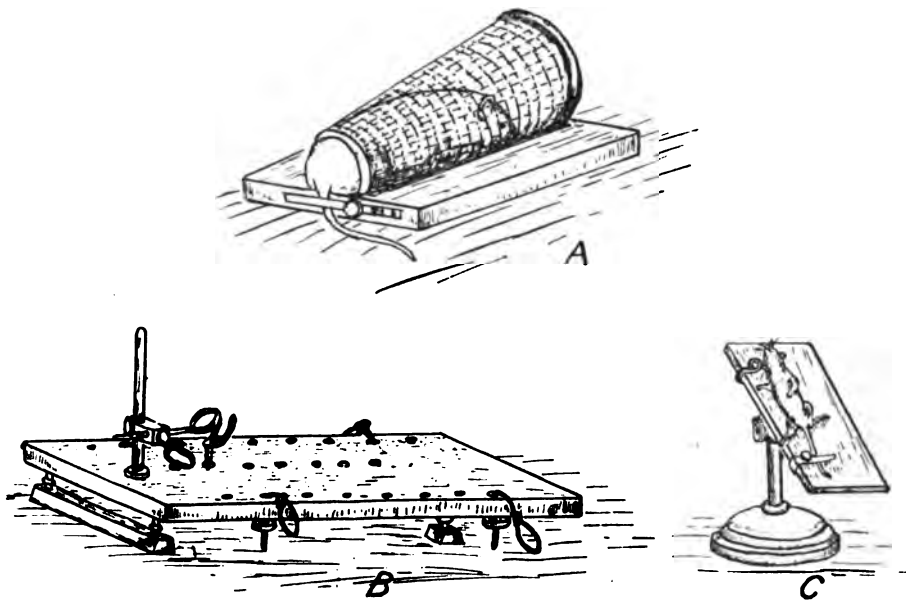


FIG. 68.—DIFFERENT FORMS OF ANIMAL HOLDERS.

In introducing a solid culture under the skin, after having cleansed the surface as in the previous description, it is necessary to cut with a sterile scissors a small fold of the skin pinched up by the fingers and then holding the edge of the incision with a sterile forceps to break a small pocket with any convenient sterile instrument in the subcutaneous tissues, into which the culture is carried on a stout platinum needle; the skin wound is then closed with a collodion dressing.

It is impossible, without some previous knowledge as to the degree of virulence of a bacterium, to determine precisely the dosage of the material to be inoculated into an animal; however, as a general rule for subcutaneous injections it is customary to employ about one per cent. of the body weight of the experiment animal of an active bouillon culture.

Intravenous Inoculation.—This is generally performed on rabbits, the vein along the posterior border of the ear of this animal being an easy one for entrance

with a fine hypodermic needle. The animal as a rule requires little holding. The needle is introduced from the dorsal side of the ear. It is scarcely necessary to shave the little hair from the skin, which is, however, to be well washed to begin with; where the hair is at all marked, however, it is best to shave the ear and wash it thoroughly. One must usually compress the base of the ear in order to make the vein in its course prominent; a small rubber band about the root of the ear serves this purpose well. The fluid in the syringe is carefully brought to the end of the needle so that no bubbles of air will be carried in the process into the circulation. The needle is then introduced into the vein as near the tip of the ear as practicable where the vein is straightest in its course, care being taken that it really enters into the interior of the vessel. The fluid is then forced gently and steadily into the vein, blanching its previous red line as the blood is displaced. Before the needle is withdrawn the pressure at the base of the ear should be released; and after withdrawal the tiny opening may be sealed with collodion. A similar operation may be practised in case of the veins of a dog's ear; in other animals and in deeper seated vessels it is necessary to expose the vein by definite dissection.

Intraperitoneal Inoculation.—This may be performed with the hypodermic syringe; or an opening may be made by regular surgical operation and through this a solid material introduced into the cavity.

The first procedure is relatively simple. The animal is fastened upon one of the mechanical holders, the hair or fur removed from a small area of the abdominal surface by means of the clippers and razor, and the surface well washed. The needle of the syringe holding the inoculating material is inserted under the skin as in subcutaneous injections, and then the abdominal wall is firmly pinched up into a comparatively large fold, this practice removing the danger of puncturing the intestine. The needle is next thrust well into the fold until its point is felt to be free in the cavity, when the fluid is forced into the latter. The needle is now withdrawn and a drop of collodion applied to the skin puncture (which is not in exact correspondence with the puncture into the cavity, the latter being protected by the skin at a little distance from the point of entrance into the cutaneous surface).

In opening the abdomen for the introduction of solid infections the animal is similarly prepared. The skin having been freed from hair and fur and the surface cleansed, the site of operation is anesthetized by cocaine, after which an incision is made through the skin with a sterile blade or scissors and the skin reflected from the underlying tissues. The incision is to be made in the median line in order to minimize the danger of bleeding. The tissue of the full thickness of the exposed abdominal wall is now pinched up into a fold by the fingers and several sutures corresponding to the size of the opening to be made are inserted through the entire thickness, passing in and out of the peritoneum in their course; these sutures are to be made transversely to the line of incision in making the opening. This done, a small incision is cut with a sterile instrument so as to open the cavity and through this the inoculation material introduced. The opening through the wall is now closed by adjustment of the sutures; after which the skin is drawn into place and closed by means of a second suturing and the line of the skin wound sealed with a collodion dressing.

Other Forms of Inoculation.—Inoculations are sometimes made into the *anterior chamber of the eye*, where the development of the consequent local lesion may be watched. In this case the lids are held by proper retractors and the surface of the conjunctiva irrigated with a solution of bichloride of mercury and afterward with sterile water; after which, with a sterile cataract needle, the cornea along the junction with the

sclera is incised and through the opening thus made the infection introduced by syringe or on a small sterilized forceps.

Inoculations *into the cerebro-spinal area* are made after performance of a definite surgical opening of the cranium by trephine or chisel, the operation being performed somewhat to one side of the sagittal line in order to avoid the longitudinal sinus. Through this opening a sterile forceps grasps the dura mater and draws it slightly upward in a small fold as well as may be done; through the fold or the adjacent tense part of the membrane the needle of the syringe is passed into the arachnoid space, into which the fluid and its contained bacteria are forced slowly; the trephine opening is covered by the replacement of the reflected overlying tissues and the external wound dressed as in ordinary surgery.

In *inhalation inoculations* the animal is placed in a small closed chamber, into the atmosphere of which the fluid in which the bacteria are suspended is vaporized mechanically, and the animal left in the chamber for a time to breathe the air contaminated with the infection.

Alimentary inoculation is accomplished by mixing some of the culture with the food and then allowing the animal to feed upon it. Occasionally enemata containing the infected matter are thrown into the lower intestinal tract.

After inoculation has been performed the experiment animals should receive regular and frequent observation as to the condition of the inoculation site, the presence of other apparent structural alterations, and the presence of any general or special symptoms. The temperature of the animal should be taken by rectum at regular intervals and charted, the movements, attitude, appetite, excretions, and the general appearance and habits of the animal watched and recorded; and the occurrence of spasms, lethargy, or other appreciated phenomena should be made a matter for record. If one be studying an organism derived from a diseased individual, all these peculiarities are to be carefully compared with the symptoms observed in the original disease and their similarity or identity established; but it is to be recollected that the difference in the experiment animal from the originally diseased individual and the mode of artificial inoculation may make considerable difference in the clinical pictures of the two cases.

Disease production by bacteria may be brought about through several possible separate or combined influences. They may (a) cause irritation by their presence or their active movements in the tissues in some locality (or if numerous points of irritation be induced, a more or less general inflammatory condition may be present); (b) they may act untowardly by the abstraction of important elements, as oxygen, from the system (either the bacteria or their products); (c) when in great numbers in some passageway, as a small vessel, they may cause its obstruction and thus perhaps interfere with nutrition or some other important function; and (d) they may elaborate or cause the formation from the body-elements of principles which are poisonous to the host (*vide* Lesson VII, section Alkaloidal Products). The most important of these modes of operation is the last, to which are due principally the general symptoms of disease, as well as the various degenerative changes of the tissues; to the first of these influences are due in large measure the inflammatory changes met about the site of inoculation.

After the death of the animal (which may be killed upon definite development of evidence of the presence of disease) a careful autopsy should be made in order to establish the alterations which have been produced in the tissues and to render certain that the organisms inoculated have been the true or probable cause of the disease

following the inoculation (this is indicated by their discovery in isolated condition in increased numbers in the body of the animal). This last constitutes the fourth of Koch's postulates for the proof of the causal relation of a given germ to disease: "*From the fluids and tissues of the experimentally diseased animal the same organism inoculated must be recovered.*"

The autopsy should be conducted as soon after death as possible, lest contaminating organisms from the intestinal canal or putrefying germs find their way into the tissues and fluids of the body; if it is impracticable to perform the operation at once, the dead animal should be refrigerated in order to prevent such occurrence. In conducting the autopsy the utmost care to prevent contamination must be observed. The usual preliminary observations as to the body, general appearance, external lesions and marks, body weight, degree of nutrition, temperature, rigidity, condition of site of inoculation, etc., are first made. The animal is then laid on its back on a convenient board and the legs extended and fastened to the board by means of tacks or staples. The skin is well wet with a solution of bichloride of mercury, and, a median incision having been made from the pubic region to the throat, the skin is dissected well off the abdomen and thorax and tacked out on the board so as to remove the likelihood of accession of bacteria from it to the parts to be exposed. The site of injection of the infection and any other subcutaneous lesions are now inspected, the removal of the skin affording opportunity for close observation; and from the inoculation site are made a number of inoculations on various nutrients, smears on glass slides are prepared for microscopic examination, and bits of the tissue are placed in absolute alcohol, where they are fixed for section-making. The body cavities are now opened by the usual longitudinal incision in the median line, with such additional lateral incisions as may be required for inspection of the interior, with a sterile pair of scissors (boiled and kept in boiled water). On opening the cavities, inoculations and smears for microscopic examination are made from any exudate encountered; and subsequently inoculations are to be made and smears prepared on glass slides from each of the important organs and the blood as detailed in Lesson V. Likewise, small bits of tissue from all suspicious points and the principal organs are fixed in absolute alcohol for sections. The remains of the animal should then be cremated, the autopsy board well disinfected, and the hands of the operator disinfected and well washed with soap and water. (In case of severe infections it is a wise practice to have the hands incased in rubber gloves during the above manipulations.)

The subsequent study of the material thus obtained concerns the discovery of the organisms in the film preparations and in the sections of the tissues, their isolation from some or all of the points from which inoculation to nutrient media was made (by culture of the inoculated media), and the recognition of destructive, degenerative, or inflammatory changes in the various structures; all of which are to be compared with the alterations known to have been produced by the original disease in case the infection was derived from a diseased individual.

Should the experiment animal or animals have recovered from the effect of the inoculation, after some days it is well to repeat the same with a view of recognizing any degree of immunity which may have been acquired from the original attack. We are accustomed to speak of the natural protection possessed by an individual against the effects of a given virus as his *natural immunity* against that germ, contrasting with it the protection gained by artificial means or through a prior attack of the same disease (*acquired immunity*). Natural immunity against the invasion and influences of bacteria in general depends on one or a number of the following factors: (a) The

protection against invasion of the germs afforded by the skin, mucous membranes, and other protective surfaces; (b) the action of the cilia present on the cells of certain membranes tending to expel the organisms seeking entrance; (c) the influences of the various secretions with which the bacteria may come in contact, as the acid juice of the stomach or the alkaline juice of the small intestine; (d) the destructive (phagocytic) action of the white blood-cells and the embryonic connective-tissue corpuscles; (e) the influences of certain agents antagonistic to bacterial development or their products, present naturally in the fluids of the body (*alexins* or *natural antitoxins*). It is believed that if these influences be sufficiently strong an invading organism is destroyed without being able to manifest its presence by the production of noticeable disease; and the limitation of the ordinary acute infections is looked upon as being due to the development of sufficiently strong action of the phagocytes, alexins, or antitoxic substances formed in the system of the diseased individual during the course of the infection. (Possibly, also, the exhaustion of some substances necessary for the growth of the infection may aid in this limitation of the disease course.)

Should the protection thus brought about be complete, so as to entirely prevent the reacquirement of the disease, such acquired immunity is said to be *absolute*; if, however, it permit the return of the disease, only in a less degree of severity, it is said to be *partial*. Advantage is taken of the possibility of acquiring such protection to induce it artificially (a) by "*inoculation*" with the natural virus of the disease in question when known to be of a mild type; (b) by introducing the virus after it has in some way been purposely weakened and is unable to induce the severe manifestations of the disease, but is yet able to confer the desired immunity (*vaccination*); or (c) by the introduction into the system of *antitoxic material* derived from individuals previously influenced by the disease itself or by the filtered toxins of the disease (as in the use of diphtheria antitoxin obtained from horses which have been subjected previously to the filtered toxins of cultures of *Mycobacterium diphtheriae* until they no longer react to large doses injected into them). The immunity acquired by such methods is spoken of as *artificial immunity*; other means are now and again available, as the influence of drugs, or rarely, the antagonism exhibited by some associated organism to the development or effects of the virus in question. The immunity which is acquired by an attack of disease (either the severe natural affection, or the mild natural disease transmitted to the individual in the practice of "*inoculation*," or the purposely weakened disease induced in vaccination) is likely to be more or less persistent, the antitoxic substances upon which it depends being produced within the system of the individual and in some instances continued for an indefinite period after the attack; such immunity is said to be an *active immunity*. When, on the other hand, the protection is afforded by the introduction of immunizing substances, as antitoxin, from another individual, or of drugs, into the system, the effect lasts only during the persistence of the dose administered; when the material has been excreted or destroyed in the system, the protection no longer exists. Such immunity is spoken of as *passive*.

Note.—The following exercises, for the sake of time, should have been inaugurated one or two weeks before the close of the class work; and provision for them should be made as soon as the class has come to understand the conditions of bacterial culture.

Exercise 81.—Each two students working together inoculate intravenously a rabbit with *M. pyogenes aureus*. In fatal cases let formal

autopsy be conducted, and from the metastatic abscesses let nutrient tubes be inoculated and films prepared for microscopic examination. In young animals note the occurrence of osteomyelitis.

Exercise 82.—Before the class let the instructor inoculate several guinea-pigs with a bouillon culture of *Bact. anthracis* grown for three weeks at a temperature of 42° C. Let the animals be exhibited to the class during the period of reaction and after recovery. After recovery let them be inoculated with a bouillon culture of the same organism grown at the same temperature for two weeks; and after recovery from this let the same operation be performed upon them with a similar culture grown for but one week at the same elevated temperature. After several days from the last inoculation let such an animal and one which has not been subjected to the vaccines be inoculated with virulent anthrax to demonstrate the acquirement of immunity by the influence of the vaccine.

Exercise 83.—Inject a guinea-pig of about two hundred and fifty grams weight with one protective unit of diphtheria antitoxin (amount of antitoxin requisite to counteract one hundred fatal doses of the toxin of the disease for an animal of such weight). Inoculate this and an unprotected guinea-pig each with 0.1 cubic centimeter of a bouillon culture of *Mycobacterium diphtheriæ* grown at body temperature for twenty-four or forty-eight hours. Compare the results. From the pig dead from the inoculation, at autopsy make smears from the site of inoculation and from the interior organs and blood. Stain these and determine the location of the germs. Make sections of the tissues and study the structural alterations evident about the site of the inoculation and in the kidneys.

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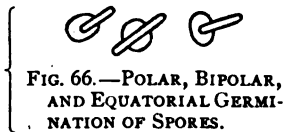
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ERRATA.

The legend for Fig. 18 should read "Culture Dish."

In legend of Fig. 40, and third line below, read for "Sedgwick-Turner," "Sedgwick-Tucker."

For Fig. 66, substitute



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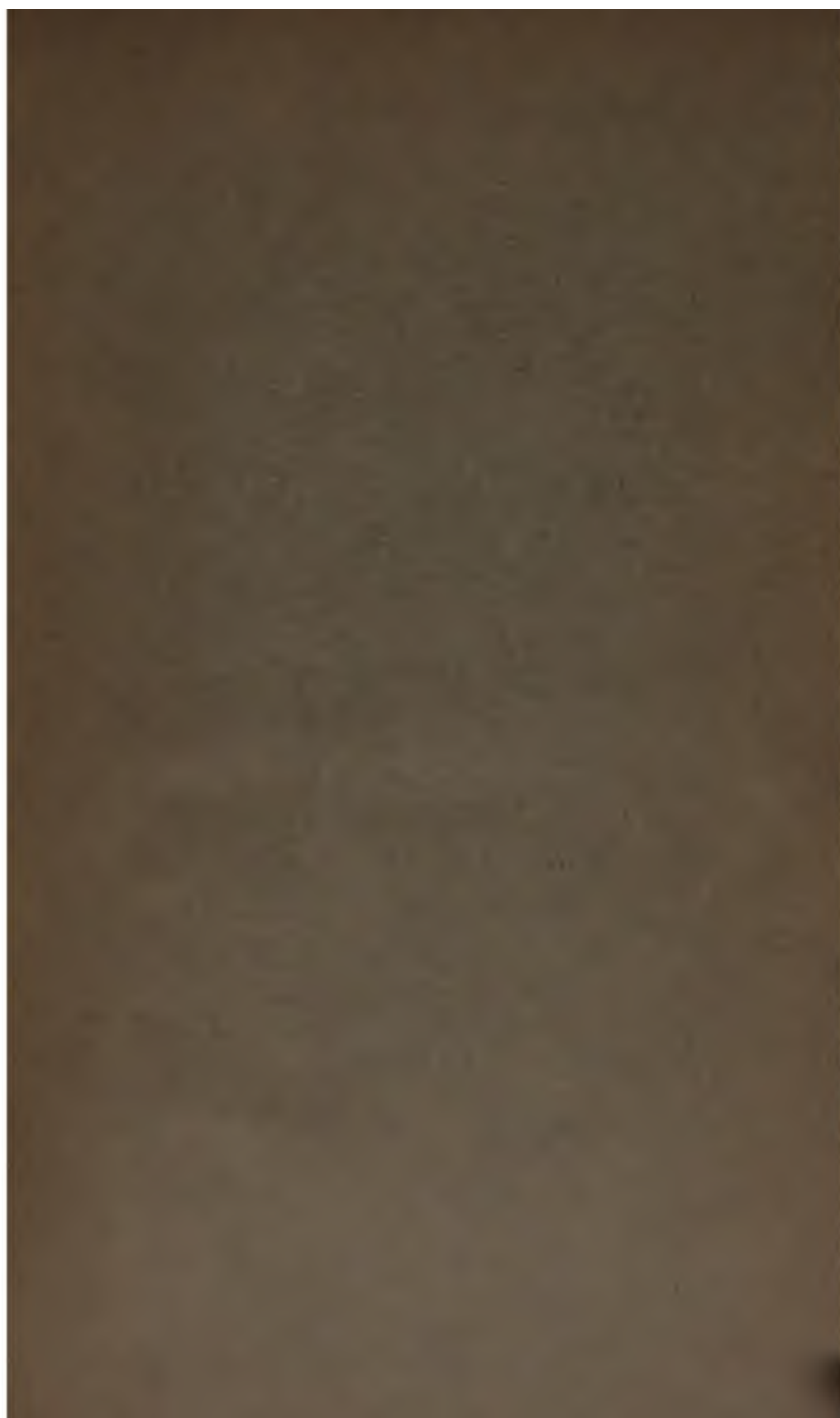
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